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DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR I

TESIS DOCTORAL

PAPEL DEL RECEPTOR CB_1 CANNABINOIDE EN EL DESARROLLO DE LA CORTEZA CEREBRAL

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2014

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RESUMEN

La corteza cerebral es la región del encéfalo que recubre los hemisferios cerebrales. Es una región funcionalmente implicada en el control de los procesos cognitivos y emocionales, y su complejidad constituye uno de los rasgos característicos de la evolución de los primates. El desarrollo de la corteza cerebral es un campo de estudio fascinante al que han dedicado sus esfuerzos numerosos científicos de ayer y de hoy. Además del interés puramente científico, la investigación acerca de los procesos que regulan el desarrollo cortical es un paso esencial para la comprensión de la etiopatología de diversas enfermedades con un origen en el neurodesarrollo (Geschwind and Rakic, 2013). En la corteza cerebral de los mamíferos encontramos diversos tipos de células neuronales y gliales. La corteza está organizada en seis capas, cada una de las cuales caracterizada por una composición celular ligeramente distinta y, por tanto, por un patrón de conectividad (aferencias y eferencias) diferente (O'Leary et al., 2007). La formación de la corteza cerebral comprende i) la generación de las células que la componen, principalmente neuronas y glía, a partir de células precursoras; ii) la especificación y la diferenciación de estas células, un proceso que culmina con la adquisición de las características moleculares y competencias funcionales distintivas de cada célula neural. Este proceso sucede habitualmente de forma concomitante a iii) la migración de las células neurales hasta su emplazamiento definitivo en el cerebro adulto. Por último, iv) se establecen las conexiones celulares que constituyen el sustrato de la comunicación nerviosa (Franco and Muller, 2013).

La formación de la corteza cerebral es un proceso exquisitamente controlado por multitud de sistemas regulatorios. El desarrollo de cada tipo de célula cortical está gobernado por sistemas "intrínsecos" encargados de la regulación de la expresión de genes que controlan la adquisición gradual de las propiedades morfológicas, moleculares y funcionales que caracterizan a su linaje. Estos programas intrínsecos son modulados por señales procedentes del nicho que contribuyen a la coordinación de los procesos mencionados. Entre los mensajeros extracelulares implicados en esta tarea se encuentran los ligandos

endocannabinoides. Se trata de moléculas de naturaleza lipídica, que actúan a través de receptores específicos, de entre los que destaca por su abundancia e importancia en el sistema nervioso central el receptor cannabinoide CB_1. El denominado sistema endocannabinoide (SEC), compuesto principalmente por los receptores cannabinoides, sus ligandos y las enzimas encargadas de su metabolismo, se expresa abundantemente en el sistema nervioso de los vertebrados, donde desempeña un papel neuromodulatorio esencial (Piomelli, 2003). El trabajo previo de nuestro grupo, así como de otros, ha demostrado que, además, el SEC está presente durante el desarrollo del sistema nervioso (Galve-Roperh et al., 2013). Las funciones atribuidas a este sistema de comunicación celular comprenden desde la regulación de la proliferación de las células precursoras neurales hasta el correcto establecimiento de las conexiones sinápticas entre distintas regiones del cerebro (Diaz-Alonso et al., 2012b). Sin embargo, los mecanismos moleculares implicados, así como el impacto que la manipulación genética y farmacológica del SEC ocasionan en el desarrollo cortical continúan siendo en gran medida desconocidos. Por tanto, para la realización de esta Tesis Doctoral nos planteamos el estudio del papel regulador que el receptor cannabinoide CB_1 ejerce sobre algunos procesos fundamentales en el desarrollo de las neuronas piramidales de la corteza cerebral. Concretamente, nos planteamos los siguientes objetivos:

- **Objetivo 1.** Explorar el papel de la señalización a través del receptor CB_1 en la regulación de la proliferación e identidad de los precursores neurales del telencéfalo dorsal, así como los mecanismos moleculares implicados. Relacionado con los resultados que componen el capítulo 1.
- **Objetivo 2.** Investigar, desde una perspectiva molecular, anatómica y funcional, la influencia que la señalización a través de CB_1 ejerce en el desarrollo de las neuronas de proyección corticofugal. Estudiar el impacto que la manipulación genética y farmacológica de la actividad de este receptor tiene sobre la generación de dichas neuronas y su función en el cerebro adulto. Relacionado con el capítulo 2.

- **Objetivo 3.** Caracterizar el papel regulador del receptor CB_1 en la migración de las neuronas piramidales corticales, identificando los mecanismos moleculares implicados. Relacionado con el capítulo 3.

Estos tres objetivos fueron abordados mediante el empleo de multitud de técnicas moleculares, celulares y comportamentales, principalmente. Combinamos la versatilidad de los cultivos celulares con la relevancia de los datos obtenidos en animales de experimentación, siempre observando estrictas normas de bienestar en estos últimos. Los resultados obtenidos han sido compilados en tres bloques, que guardan relación con los tres objetivos principales planteados. Parte de las evidencias experimentales que constituyen estos tres capítulos han sido publicadas, mientras que otras se encuentran aún en fase de preparación. Estos resultados pueden resumirse en:

- **Capítulo 1.** En este capítulo analizamos, en primer lugar, la expresión y funcionalidad del receptor CB_1 en las células precursoras de las neuronas de proyección de la corteza cerebral. Estudiamos a continuación el papel que la señalización a través del receptor CB_1 cumple en la expresión y la función de varios factores de transcripción requeridos para la regulación de la identidad y capacidad neurogénica de estos progenitores. En concreto, describimos el mecanismo mediante el cual la señalización vía CB_1 en células de glía radial (principal reservorio de precursores de neuronas piramidales corticales) modula la actividad del factor de transcripción Pax6, a través de un mecanismo dependiente de la vía de supervivencia PI3K/Akt/mTORC1. Este mecanismo contribuye a la generación de progenitores intermedios o amplificadores y, por tanto, a la generación de la diversidad neuronal de la corteza cerebral. Estos resultados están publicados en el siguiente artículo:

  Díaz-Alonso J, Aguado T, de Salas-Quiroga A, Ortega Z, Guzmán M, Galve-Roperh I. *"CB_1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex"*. Cereb Cortex. 2014 Mar 7. [Epub ahead of print]

- **Capítulo 2.** En este bloque de resultados se describe la regulación que ejerce el SEC en la generación y diferenciación de las neuronas de proyección cortico-espinal (CSMN). Describimos el mecanismo a través del cual la señalización a través de CB_1 contribuye a orquestar el balance entre varios factores de transcripción que identifican y especifican a las distintas subpoblaciones de neuronas piramidales corticales. En concreto, este mecanismo opera atenuando la acción del represor transcripcional Satb2 sobre el promotor de Ctip2, un elemento regulador requerido para la correcta especificación de estas neuronas. Observamos que en ratones deficientes en el gen que codifica el receptor CB_1 tanto el desarrollo como la función de estas neuronas en el animal adulto está severamente afectada. Además, en ratones expuestos a Δ^9-THC durante el desarrollo prenatal se recapitulan las alteraciones observadas en los ratones $CB_1^{-/-}$. Estos resultados constituyen un artículo publicado y otro en preparación:

  Díaz-Alonso, J, Aguado, T., Wu, C-S., Palazuelos, J., Hofmann, C., Garcez, P., Guillemot, F., Lu, H.C., Lutz, B., Guzmán, M., Galve-Roperh, I. *"The CB(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the Ctip2/Satb2 transcriptional regulation axis"* J. Neurosci. 32(47):16651-65 (2012)

  Díaz-Alonso, J*, de Salas Quiroga, A*, Vega, D, García-Rincón, D, Lutz, B, Guzmán, M, Galve-Roperh, I. *"Impaired corticospinal motor neuron development and function and increased seizure susceptibility in prenatally Δ^9-tetrahydrocannabinol-exposed mice"* En preparación. *Igual contribución

- **Capítulo 3.** En este capítulo investigamos el papel de la señalización a través del receptor CB_1 en la regulación de la migración radial de las neuronas piramidales corticales durante el desarrollo. Mediante la manipulación local de la expresión génica a través de la electroporación intraútero de diversas construcciones, observamos que la pérdida de función del receptor CB_1 ocasiona el bloqueo de la migración de las neuronas recién generadas. También describimos que el receptor cannabinoide señaliza promoviendo este proceso mediante la modulación de la actividad de RhoA, una proteína

reguladora del citoesqueleto de actina. Este trabajo se encuentra en vías de preparación en este momento:

Díaz-Alonso, J, de Salas-Quiroga, A, Garcez, P, García-Rincón, D, Guillemot, F, Guzmán, M, Galve-Roperh, I. *"The CB_1 cannabinoid receptor signals radial migration of pyramidal neurons in the developing mouse cortex through the inhibition of RhoA".* En preparación.

En líneas generales, en este trabajo hemos tratado de expandir el conocimiento existente sobre los mecanismos a través de los cuales el SEC participa en la regulación de varios procesos que tienen lugar durante el desarrollo de la corteza cerebral. La expansión de los precursores de las neuronas piramidales corticales, su posterior especificación y diferenciación, y la migración de las neuronas recién generadas hasta alcanzar su posición final en la corteza constituyen eventos clave en la ontogenia de la corteza cerebral. La aparición de un número creciente de enfermedades neuro-siquiátricas se relaciona con el mal funcionamiento de estos mecanismos (Pang et al., 2008). En este sentido, hemos tratado sistemáticamente de dilucidar las consecuencias que alteraciones en el funcionamiento del SEC durante el desarrollo pueden tener sobre la apropiada formación de la corteza cerebral, y proponemos dirigir futuras investigaciones hacia la posible intervención sobre el SEC como aproximación terapéutica en estas patologías. Las conclusiones de esta Tesis doctoral, siguiendo el orden de los objetivos y resultados presentados, son las siguientes:

Capítulo 1.

- El receptor cannabinoide CB_1 se expresa, si bien en niveles bajos, en las células precursoras neurales del telencéfalo dorsal.
- La señalización a través del receptor CB_1 contribuye al establecimiento y mantenimiento de la identidad de los progenitores corticales, así como a la regulación de su proliferación, a través de la modulación de la actividad del factor de transcripción Pax6.
- El receptor CB_1 actúa a través de la cascada de señalización de mTORC1 para promover la actividad transcripcional de Pax6 y, por consiguiente, la expansión de la población de progenitores corticales intermedios.

Capítulo 2.

- La señalización a través del receptor cannabinoide CB_1 atenúa la actividad del represor transcripcional Satb2, participando de este modo en el control de la especificación de las neuronas piramidales corticales.
- La señalización endocannabinoide se requiere para la apropiada generación y especificación de las neuronas motoras cortico-espinales y, por tanto, para la función motora fina en el cerebro adulto.
- La disrupción de la señalización a través del receptor CB_1 ocasionada por la exposición prenatal a Δ^9-tetrahidrocannabinol (THC) impide el normal desarrollo de las neuronas motoras cortico-espinales y, consecuentemente, su función en el cerebro adulto.
- Las alteraciones en el desarrollo del sistema nervioso causadas por la exposición a THC durante la gestación convergen en un incremento en la susceptibilidad a la epilepsia.

Capítulo 3.

- El receptor cannabinoide CB_1 promueve la migración radial de las neuronas piramidales hacia la corteza cerebral. Este efecto pro-migratorio se fundamenta en la modulación de la actividad de la proteína reguladora del citoesqueleto de actina RhoA. Además, el ligando endocannabinoide 2-araquidonoilglicerol actúa como factor quimioatrayente para estas neuronas.

ABBREVIATIONS

2-AG: 2-Arachidonoylglicerol.
AEA: Anandamide
Akt: murine thimoma viral oncogene homolog.
APN: associative projection neuron
BDNF Brain derived neurotrophic factor
bHLH Basic helix-loop-helix
BMP: bone morphogenetic protein
CB_1: cannabinoid receptor, type 1
CB_2: cannabinoid receptor, type 2
CBD: cannabidiol
CFuPN: corticofugal projection neuron
CNS: central nervous system
Coup-TFI: chicken ovoualbumin upstream promoter-transcription factor I
COX: cyclooxigenase
CP: cortical plate
CPN: callosal/commissural projection neuron
CSMN: corticospinal motor neuron
CThPN: corticothalamic projection neuron
Ctip2: Coup-TF interacting protein 2
DAGL: diacylglicerol lipase
eCB: endocannabinoid
ECS: endocannabinoid system
EGF: epidermal growth factor
Emx2: empty spiracles homeobox protein 2
ERK: Extracellular signal-regulated kinase
FAAH: fatty acid amide hydrolase
Fezf2: FEZ family zinc finger 2
FGF: fibroblast growth factor
GABA: γ-aminobutyric acid
GPCR: G protein-coupled receptor
IPC: intermediate progenitor cell

IZ: intermediate zone

MAPK: mitogen-activated protein kinase

MAR: matrix attachment region

MGL: monoacylglicerol lipase

MZ: marginal zone

NAPE-PLD: N-acyl phosphatidylethanolamine-phospholipase D

NEC: neuroepithelial cell

Pax6: Paired box protein 6

PI3K: phosphoinositide-3-kinase

PLC: phospholipase C

PPAR: peroxisome proliferator-activated receptor

RGC: radial glial cell

Satb2: special AT-rich sequence-binding protein 2

SCPN: subcerebral projection neuron

shRNA: short hairpin ribonucleic acid

siRNA: short interfering ribonucleic acid

Sp8: specificity protein 8

SVZ: subventricular zone

Tbr2: T-box brain protein 2

TGF: transforming growth factor

THC: Δ^9-tetrahydrocannabinol

TRPV1: transient receptor potential cation channel subfamily V, type 1

VZ: ventricular zone

ABSTRACT

The endocannabinoid system (ECS) participates in the regulation of a wide array of physiological processes. A particularly crucial neuromodulatory role has been ascribed for the ECS in the mature brain, where it controls membrane excitability and neurotransmitter release. Different elements of the ECS are also present in the CNS during development, and have been shown to control important processes, including neural progenitor proliferation, fate commitment and morphogenesis. Here, we characterized the expression of CB_1 receptors in cortical progenitor cells *in vivo*, and identified a previously unknown role of this receptor in the regulation of Pax6 activity via mTORC1 pathway activation. Thus, radial glial cell self-maintenance and intermediate progenitor cell expansion were found to be controlled by CB_1 signaling. We also studied the role of the ECS in cortical pyramidal cell differentiation, and found CB_1 receptor signaling to play an essential role in corticospinal motor neuron (CSMN) specification. Furthermore, we demonstrated that endocannabinoid signaling contributes to this process by tuning the transcriptional-regulation machinery responsible for the generation of cortical pyramidal neuron diversity. We also describe an unprecedented role of the ECS in the regulation of the radial migration of newborn cortical projection neurons *in vivo*, and provide evidence supporting that the pro-migratory intracellular signaling cascade triggered by CB_1 receptor activation includes the inhibition of the cytoskeleton-regulatory protein RhoA. Last, but not least, we modeled cannabis exposure during embryonic development in mice and found overt alterations in corticofugal neuron development as a consequence of disrupted physiological CB_1 signaling. Moreover, the altered CNS development in THC-exposed mice sensitized them to suffer epileptic seizures and alteration in fine-motor behavior. Altogether, our results indicate that CB_1 receptors are present and functional during cortical development and that altered ECS signaling, both by genetic and pharmacological manipulations, impairs several steps in cortical development, thereby potentially underlying the emergence of a number of neurodevelopmental pathologies.

INDEX

1. INTRODUCTION

The cerebral cortex is the structure that covers the telencephalon. It contains hundreds of different cell types, organized in a truly sophisticated neural network, which bears the most complex tasks in the brain including,motor control, sensory perception, learning, emotional responses and, in humans and other animals, reasoning, language and conscious thinking (Franco and Muller, 2013). The cerebral cortex has been subject to intense remodeling during the 300 million years of mammalian evolution, and now constitutes a genuine hallmark of our lineage class (Geschwind and Rakic, 2013). The cerebral cortex can be defined as the most important structure in the human brain. This clearly indicates its importance orchestrating the different brain structures function. The computational power that this structure provides enables the performance of the sophisticated intellectual tasks that, in turn, can even result in the questioning of its own nature.

1. General aspects of cortical development

The ontogeny of the cerebral cortex is an extremely tightly regulated process, in which intrinsic gene expression programs and intercellular signaling converge to allow the emergence of the impressive cytoarchitecture of this organ. This matter of study has attracted the attention of scientists since ancient times, and the titanic effort of the neuroscientists of the past and recent days has pinpointed many of the processes that take place during cerebral cortical development, with exquisite detail in some cases. However, some questions still need to be answered.

Many diseases are caused by the malfunction of the cerebral cortex. Among them, some are genuinely human diseases, as they affect purely human behaviors, such as dyslexia, intellectual disability, autism spectrum disorder, attention deficit hyperactivity disorder, and schizophrenia (Preuss et al., 2004) as well as a number of human-specific neurodegenerative conditions like Alzheimer's disease (Miller et al., 2010). Others, like epilepsy, occur also in other vertebrates. A developmental origin has been ascribed for an increasing number of these pathologies (Geschwind and Rakic, 2013; Rubenstein, 2011), making research in cortical development not only exciting from a basic scientific point of view but also relevant from the clinical perspective.

To note, apart from the study of human-derived specimens, obviously limited, our knowledge about the mechanisms that govern human cortical development, and thus the basis of many cortical disorders with a developmental origin comes from studies largely involving inference from other animals, both invertebrates (fruit fly-*Drosophila melanogaster*-being the most prominent) and vertebrates (of which, quantitatively, chick, rat and mouse are the main representatives, with qualitatively crucial contributions from other mammals, specially primates other than human). For

this study, we have almost exclusively employed the mouse cortex as a model what, while obviously limiting the impact of our findings from a clinical point of view, has enabled the use of sharp and specific pharmacological and genetic tools, thus allowing the deep exploration of the processes we focused in.

1.1. Early patterning of the cerebral cortex

The cerebral cortex emerges from the prosencephalon, the anterior part of the neural tube. During early development, the dorsal ectoderm specifies to form the neural plate. Later on, in a process termed **neurulation**, the neural plate forms the neural tube, an outstretched, hollow structure, uniformly shaped along the rostrocaudal axis at the earliest stages. At E8-9 in mouse, the neural tube closure finishes (Copp et al., 2003). Then, under the influence of the signals from the **organizer** and the **anterior visceral endoderm nodes**, the formerly uniform structure begins to suffer a rostrocaudal/anteroposterior patterning (Hebert and Fishell, 2008): several evaginations of the neural tube give rise to the so-called primary neural vesicles: the **prosencephalon**, the **mesencephalon** and the **rombencephalon**, which constitute the embryonic primordia of the **forebrain**, **midbrain** and **hindbrain**, respectively (Puelles and Rubenstein, 2003; Rallu et al., 2002). Afterwards, secondary vesicles originate from the prosencephalon, now segregated into **diencephalon** (prospective thalamic and hypothalamic nuclei) and **telencephalon**, see Fig. 1.

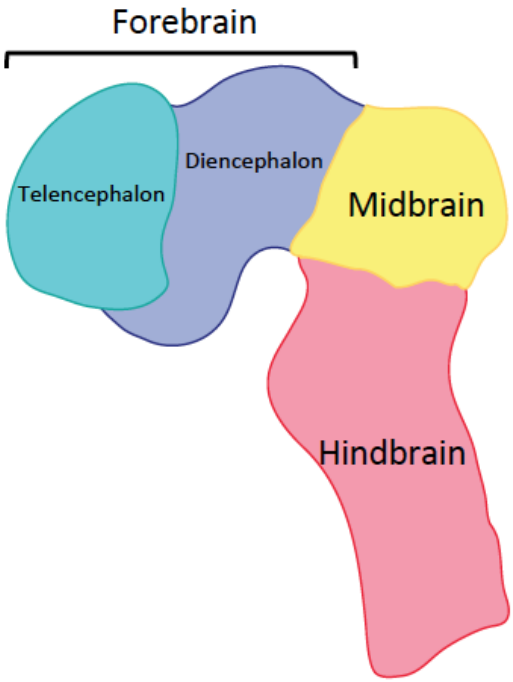


Figure 1. **Early patterning of the CNS**. *The initial subdivisions of the developing CNS are depicted.*

The telencephalon is further subdivided into ***pallium*** (dorsal telencephalon) and ***subpallium*** (ventral telencephalon), under the shaping force of the dorsoventral patterning cues (Rubenstein et al., 1998), see Fig. 2. Starting around E10-11, the *subpallium* specializes into three vesicles: the lateral, medial and caudal **ganglionic eminences**. These territories are the prospective basal ganglia, and constitute the niche for the generation of the vast majority of GABAergic telencephalic neurons (Marin and Muller, 2014), see section 2. The dorsal telencephalon comprehends three distinct areas: the **neocortex** is the largest region, and is positioned between the two other regions of the cerebral cortex, the **archicortex** (entorhinal cortex, retrosplenial, subiculum, and hippocampus) and the **paleocortex** (olfactory piriform cortex) (O'Leary et al., 2007).

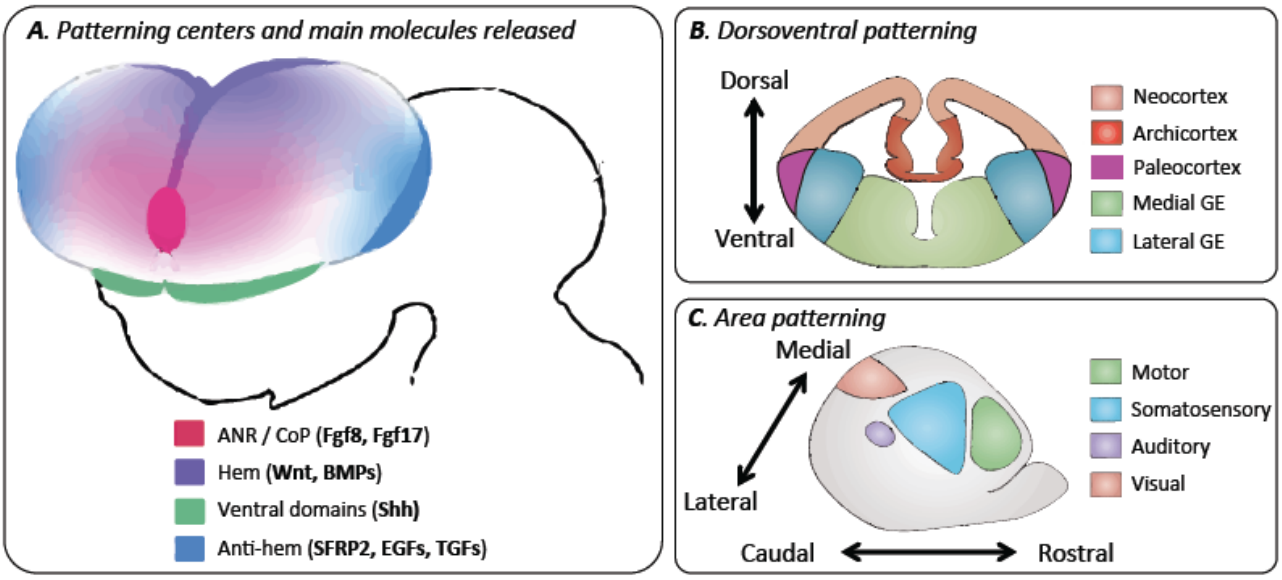


Figure 2. **Shaping the telencephalon**. *In A, the main patterning centers of the developing telencephalon, and the morphogens released from them are depicted. In B and C, the resulting dorsoventral and area patterning of the telencephalon, respectively, is illustrated. Modified from* (O'Leary and Sahara, 2008) (Hebert and Fishell, 2008) and (Greig et al., 2013).

1.2. Radial and tangential organization of the neocortex

The mammalian neocortex is organized in two axes, radial and tangential: in its tangential dimension, the neocortex is organized into '**areas**' that are functionally unique subdivisions distinguished by differences in cyto- and chemoarchitecture, input and output connectivity and gene expression patterns (O'Leary and Sahara, 2008). There are four primary cortical areas, three of them are committed to the processing of sensorial inputs (visual –**V1**-, auditory –**A1**- and somatosensory **-S1**-) and one to the motor controlling outputs (**M1**) (O'Leary et al., 2007), Fig. 2. The developmental process that segregates the cerebral cortex into functional areas is called **arealization**, and it relies on the existence of several patterning centers in the edges of the developing telencephalon. These patterning nodes release **morphogens** (**FGFs**, **Shh**, **BMPs** and others) that generate a gradient expression of several transcription factors in cortical progenitors. These transcription factors (**Emx2**, **Pax6**, **Sp8**, **COUP-TFI**, among others) trigger then distinct gene expression programs, causing the progenitor cells (and their progeny) to gradually acquire their differential properties (section 5.1.). The arealization process occurs gradually, so the limits between adjacent prospective areas, initially smooth, become increasingly sharp throughout development. This process is greatly conserved among different mammalian families, suggesting a common genetic program orchestrating cortical patterning. In addition, the plasticity of the wiring of the cerebral cortex, exemplified by its re-wiring upon traumatic injury or damage in the sensory pathways suggests the existence of certain degree of freedom in the govern of this process (Alfano and Studer, 2013).

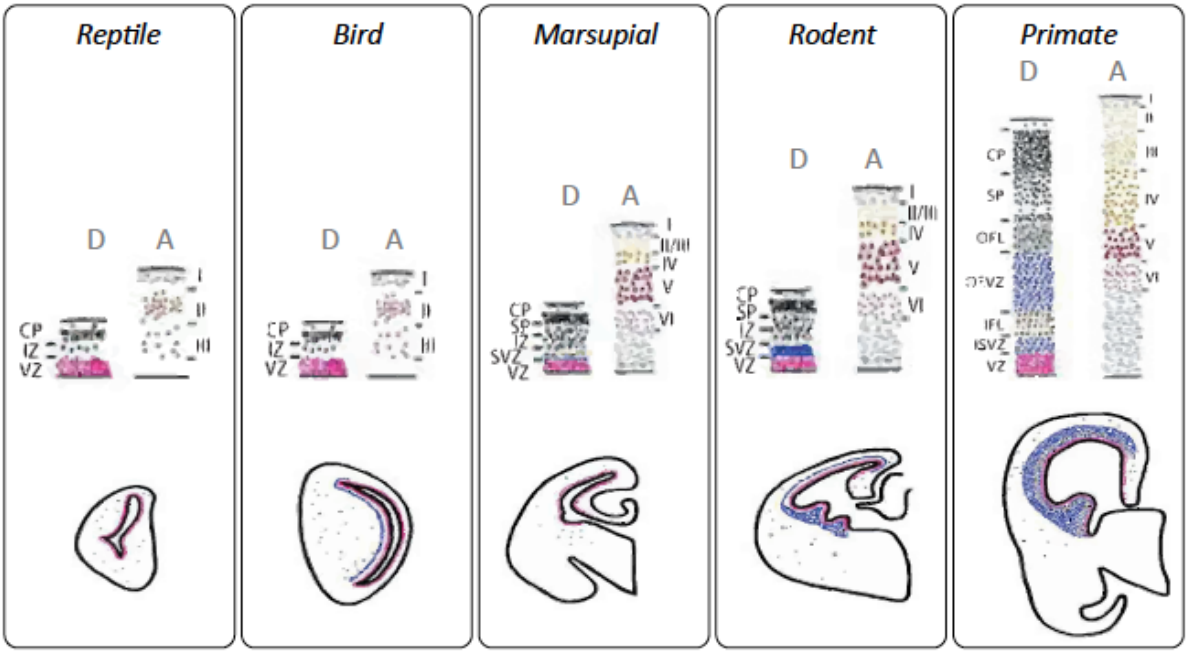


Figure 3. **Evolution of the cerebral cortex laminar organization**. *Developmental (*D*) and adult (*A*) laminar composition of the cerebral cortex in reptiles, birds, marsupials, rodents and primates is illustrated. Modified from* (Molnar, 2011).

Radially, the mammalian neocortex can be divided into six morphologically distinct **layers** (**I to VI**) that contain different neuronal populations attending, besides other considerations, to their connectivity, gene expression pattern and birthdate (Molyneaux et al., 2007). The 6-layered neocortex is the philogenetically most recent feature of the mammalian brain and, interestingly, constitutes a distinctive mammalian hallmark, as in the other amniotes' cerebral cortex only 3 layers are distinguishable, corresponding to mammalian layers I, V and VI (Cheung et al., 2007; Puelles et al., 2000), Fig. 3.

The understanding of the radial and the tangential organization of the cerebral cortex led to the proposal, decades ago, of the **radial unit hypothesis** (Mountcastle, 1997; Rakic, 1988), elegantly bringing together the empiric data on the two organizational axes of the cortex. This theory sustains that "*the ependymal layer of the embryonic cerebral ventricle consists of proliferative units that provide a proto-map of prospective cytoarchitectonic areas. The output of the proliferative units is translated via glial guides to the expanding cortex in the form of ontogenetic columns, whose final number for each area can be modified through interaction with afferent input*"(Rakic, 1988), Fig. 4. This model, complemented and amended by more recent discoveries enabled by novel tools, is still in force nowadays. Now we know that the neurons derived from a radial glial cell are not completely fixed to a given column and, instead, have certain degree of freedom to spread laterally and integrate in neighboring columns, in a process that seems to be governed by Ephrins and Eph receptors (Dimidschstein et al., 2013; Torii et al., 2009)).

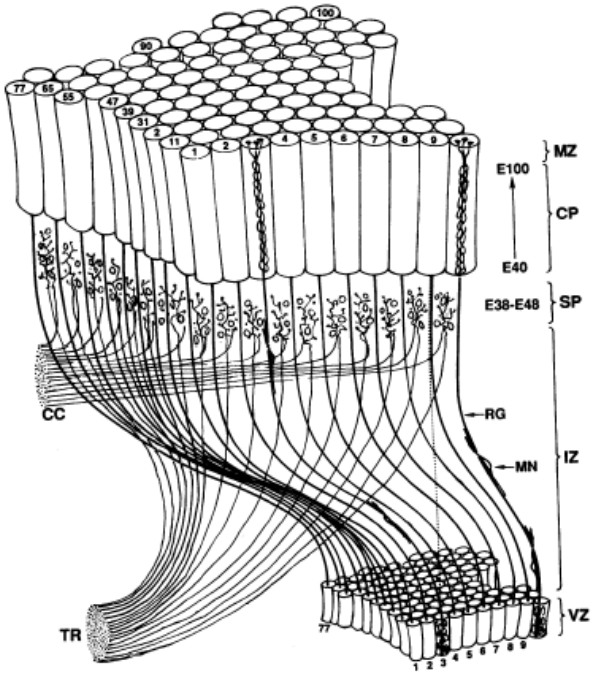


Figure 4. **The radial unit hypothesis***. From* (Rakic, 1988)

2. Main types of cells in the cerebral cortex

The cells populating the cerebral cortex can be divided into two major classes: neurons and glia. Cortical glial cells are also classified into two major classes: macro and microglia. The former division is comprised by astrocytes and oligodendrocytes, both originated in the **ventricular and subventricular zones** (**VZ and SVZ**) of the developing cerebral cortex; the latter, by myeloid cells of a mesodermal origin (Wake et al., 2013). Depending on their connectivity pattern and neurochemical profile, cortical neurons belong to either the glutamatergic (excitatory) **projection neuron** or to the GABAergic (inhibitory) **interneuron** lineages. Both main types of cortical neurons can be further divided into several sublineages. Interneurons represent approximately 20% of cortical neurons, and are classified into 19 different subclasses, attending to their morphological, molecular and phisiologycal features (Petilla Interneuron Nomenclature et al., 2008) (DeFelipe et al., 2013). On the other hand, pyramidal neurons account for the remaining 80% of cortical neuronal cells, and are also classified into different groups, mainly attending to their connectivity pattern and their position within a certain cortical layer. However, the **hodological** and **laminar** criteria are likely insufficient to define all subtypes of cortical pyramidal neurons, as neurons with similar projection patterns are often dispersed across multiple layers (Fame et al., 2011) and, moreover, different subsets of neurons with diverse molecular characteristics are often found within a single layer (Franco and Muller, 2013).

Our understanding about the generation of the great diversity of cortical neurons largely relies on studies determining the identity and behavior of their precursor cells. Hence, we know that pyramidal neurons and interneurons originate in different neurogenic niches: cortical interneurons are born in subpallial localizations, mainly the medial and caudal ganglionic eminences, so they need to undergo a long **tangential migration** to finally reach the dorsal telencephalon and establish in their final position in the neocortex (Gelman and Marin, 2010; Marin et al., 2010; Wonders and Anderson, 2006), Fig. 5. Pyramidal neurons precursor cells are located in the dorsal telencephalic VZ/SVZ, thus they undergo a significantly shorter **radial migration** process towards their final destination in the cortical plate (Nadarajah et al., 2003), Figs. 5, 12. Given that this Thesis mainly

focuses in pyramidal neuron ontogeny, interneurons will only be taken into consideration if they are involved in important processes affecting pyramidal cell generation and/or maturation.

Both cortical projection neurons and interneurons are affected by the columnar cortical framework (Rakic, 2006). This seems to happen also for astrocytes distribution, even contributing to its establishment and maintenance (Colombo and Reisin, 2004). Thus, each cortical layer is populated by a slightly different neuronal and glial cell pool: attending only to projection neurons (PNs), **layer VI** is greatly enriched in corticothalamic PNs; **layer V**, in PNs connecting to the basal ganglia, midbrain, hindbrain and spinal cord; **layer IV** is populated by the unique stellate cells, projecting locally and **layers II and III** are vastly occupied by callosal/commissural PNs (Molyneaux et al., 2007), see Figs. 5, 13 and 14 for details. **Layer I** is occupied by **Cajal-Retzius cells**, a pioneering neuronal population that only exists transiently and whose task is to instruct a crucial aspect of cortical organization, the radial migration of pyramidal neuroblasts. probably also intervening in their specification given the reciprocal influence of these processes (Villar-Cervino and Marin, 2012; Villar-Cervino et al., 2013). A great effort has been devoted to understanding the developmental process of **cortical layering**. The emerging theory states that there are two primary requisites for the correct establishment of the columnar organization of the cortex: i) the migration of neurons and

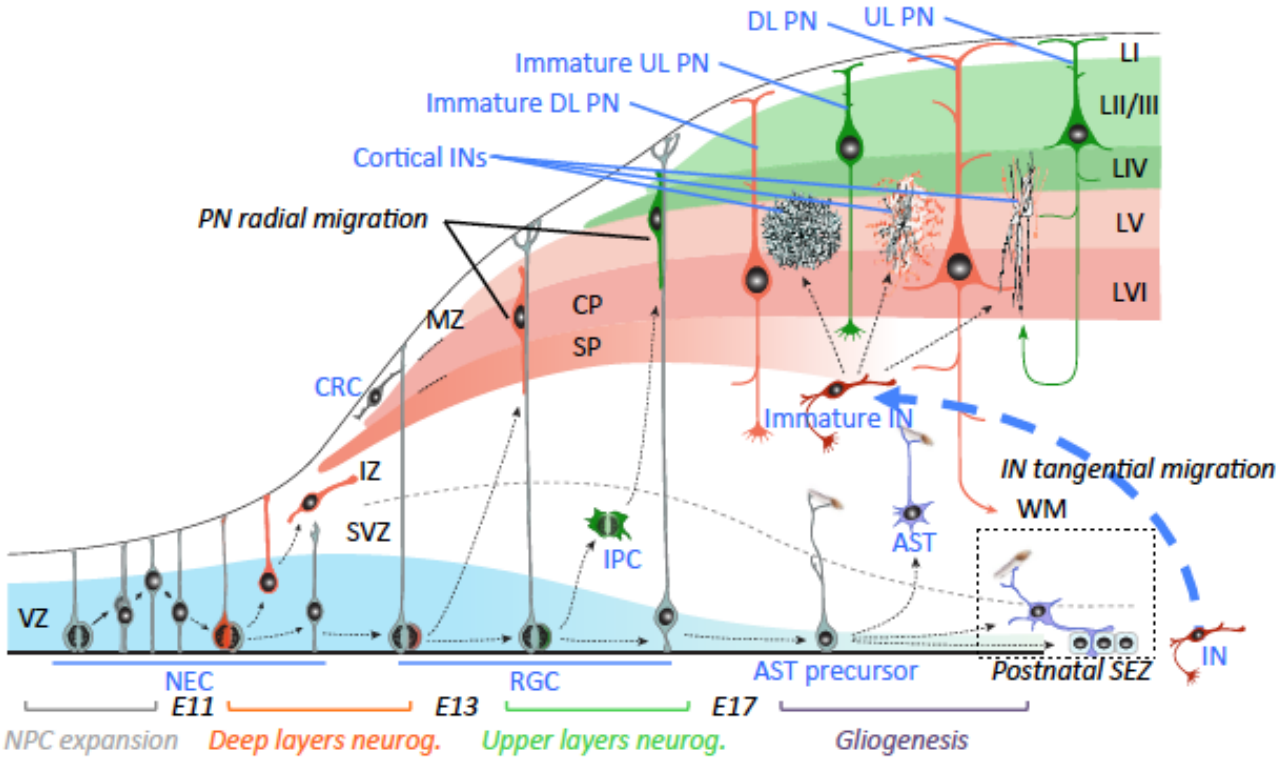
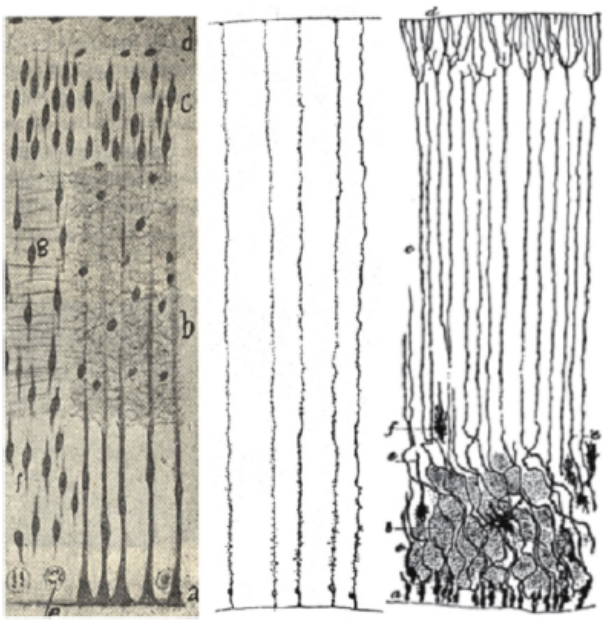


Figure 5. **Overview of cortical development**. *Main cell types, anatomic compartments and processes taking place during corticogenesis. Adapted from (Kwan et al., 2012). Abbreviations: PN, projection neuron; IN, interneuron; AST, astrocyte; NEC, neuroepithelial cell; RGC, radial glial cell; IPC, intermediate progenitor cell; PP, preplate; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; SVZ, subventricular zone; WM, white matter; SEZ, subependymal zone.*



glia to their correct final position in the cortex and ii) the acquisition of the molecular identity that distinguishes every cell type in the cortex (Kwan et al., 2012), see Fig. 5. A more detailed explanation of the cortical layering process will be given in the next sections.

Figure 6. **"A Potpourri of Classical Depiction of Neural Glial Stem Cells"** *(A) Illustration taken from the work of His (1904) on the human embryonic forebrain, including the horizontal and vertical (asymmetric) division of the mitotic cells. (B) The drawings of the "ependymal glial cells" in the human fetal cerebrum at 10 weeks old stand with the Golgi method (Retzius, 1893). (C) Epithelial (radial glial) and neuroglial cells of the cerebral cortex at later stage of development in the neonatal rabbit stained with the Golgi method depicted by Ramón y Cajal (Ramón y Cajal, 1909. Adapted from (Breunig et al., 2011).*

3. Origin and specification of cortical pyramidal neurons: building the cerebral cortex

The different cortical layers are generated by the colonization of the cortical plate by sequential neurogenic waves: at the earliest neocortical developmental stage a **preplate** emerges between the ventricular zone and the pial surface, the apical and basal edges, respectively, of the developing cortex. This preplate is composed of neurons and, likely, other cell types and, due to the arrival of the next waves of newborn neurons, is rapidly split into **subplate** and **marginal zone**, being the latter populated by the aforementioned Cajal-Retzius cells and finally constituting layer I (Fig. 5). Then, the **cortical plate** is progressively enlarged following an **inside-out** pattern (Angevine and Sidman, 1961). The first to arrive (peaking around E12) are the prospective layer VI pyramidal neurons, followed by layer V (E13), IV (E14), III (E15) and finally II (E16) (Figs. 5, 13). Once all pyramidal neurons have born, cortical progenitor cells generate only astrocytes. This process begins in the latest embryonic development stages and continues at perinatal life stages, concomitantly with the onset of oligodendrocyte generation (Miller and Gauthier, 2007; Skoff, 1990), Fig. 5. The delayed gliogenesis *versus* neurogenesis is thought to guarantee the correct establishment of the axonal connections before the glial cells add to the scaffold (Qian et al., 2000).

3.1. Pyramidal neurogenic areas in the developing neocortex

Since the fall of the 18th century, neuroscientists know that the cell divisions that generate cortical neurons occur in the zone adjacent to the ventricular walls (Breunig et al., 2011). Neuroanatomists like **Camilo Golgi**, **Santiago Ramón y Cajal** and, especially, **Wilhelm His**, described that, in the

developing cerebral cortex, mitotic figures occurred almost exclusively in the proximity of the lateral ventricles of the developing human neocortex, and even made the first insights on the coexistence of symmetric and asymmetric modes of cell division in this context (Fig. 6)

3.2. Types of neocortical progenitor cells

The emergence of the labeled analogs of thymidine (first H^3-Thimidine, then halogenated atom-labeled deoxyuridine –mainly BrdU, CldU and YdU-), allowed, in the mid 20th Century, the confirmation of the previous findings, leading to the proposal by the Boulder Committee (1970) of a uniform nomenclature substituting the former, inaccurate ones. Thus, the American Association of Anatomists accepted that the mammalian pallial **ventricular and subventricular zones** (**VZ and SVZ**) contained the sources for both neurons and macroglia in the cerebral cortex.

3.2.1. Neuroepithelial cells

Neocortical pyramidal neurons and macroglia derive from a specialized (neuro)epithelium lining the dorsal surface of the lateral ventricles, right after the neural tube closure finishes. It consists of a single sheet of the cortical primary neural stem cell, called **neuroepithelial cell (NEC)**. These cells mainly divide symmetrically to amplify the neural stem cell pool, and have unique morphological features, being the most important their apico-basal polarity: they are attached to the apical surface, where they form tight junctions with one another (Aaku-Saraste et al., 1996) and to the basal lamina, in the pial surface, by integrins (Graus-Porta et al., 2001), Fig. 7. They undergo the so-called interkinetic nuclear migration: DNA replication takes place in the most basal side of the neuroepithelial wall, while cell division occurs in the apical (ventricular) surface (Sauer and Walker, 1959). This nuclear movement gives the neuroepithelium a pseudo-stratified appearance.

3.2.2. Radial glial cells

NECs then give rise to a new type of cells, the precursors of most excitatory neurons, astrocytes and oligodendrocytes of the cerebral cortex: **radial glial cells (RGCs)**. Their nuclei are located in the VZ, derived from the previous neuroepithelium and, as their name suggest, they have a "radial" morphology: inheriting the apico-basal cell polarity from NECs, they extend two cytoplasmic processes; one –the apical- towards the ventricular wall, where the NECs' tight junctions are replaced by adherens junctions (Martynoga et al., 2012), and other –basal- to the pial surface. This morphological feature was first described by Golgi in the 19th century and corroborated recently (Gotz et al., 2002; Malatesta and Gotz, 2013). Apart from the polarity and interkinetic nuclear migration, RGCs share with NECs the expression of several markers, such as the intermediate filaments **nestin** and radial cell 2 (**RC2**) and the transcription factor **Pax6** (Gotz et al., 2002). On the

other hand, they are called "glial" owing to the expression of (astro)glial markers such as the Glial Fibrilary Acidic Protein (**GFAP**) –albeit at low levels in the case of rodents (Sancho-Tello et al., 1995)-, the Glutamate/Aspartate Transporter **GLAST** and the Brain Lipid Binding Protein (**BLBP**). Thus, morphological and molecular evidences supported the notion that, apart from constituting scaffolds aiding newly born neurons to migrate radially towards their final position in the cortex, RGCs are precursors only for cortical astrocytes, as early suggested by His, who called them "spongioblasts" in the 19th century (Alvarez-Buylla et al., 2001). It was only recently that, by using FACS-sorting and lineage-tracing experiments, these cells were finally demonstrated to generate also cortical neurons, both *in vitro* and *in vivo* (Malatesta et al., 2000; Noctor et al., 2001). They were shown to divide assymetrically to yield –directly or, much more abundantly, indirectly through the generation of an intermediate progenitor cell, a neuron and another radial glial cell (Miyata et al., 2001), Fig. 7.

3.2.3. Intermediate progenitor cells

Intermediate progenitor cells (IPCs) are a secondary progenitor pool preferentially located in the SVZ (Takahashi et al., 1995), thus basal to the RGCs pool, which inspired their alternative name, "basal progenitors". These are cells that, derived from a radial glial cell, lose their apical and basal processes and exhibit a multipolar morphology instead. However, they retain their mitotic capacity and the expression of proliferation markers such as **phosphorylated vimentin** and phospho-Histone 3 (**p-H3**) (Noctor et al., 2007). These secondary proliferative cells of the developing cortex can undergo one or more **symmetric cell divisions** (Noctor et al., 2004), what significantly increases the yield of cortical cells derived from a single radial glial cell, Fig. 7. They are characterized by the expression of several markers including, but not limited to, **Svet1** (Tarabykin et al., 2001) and **Eomes/Tbr2** (Englund et al., 2005). Of note, the expression of these factors must not be taken alone to identify an IPC, as both have been shown to be also expressed by postmitotic cells –Svet1 is intensely expressed by upper layer neurons while Tbr2 is transiently expressed by many newborn cortical pyramidal neurons-. The relative contribution of intermediate progenitors to the total number of proliferating cells in the cortex increases with time: the subventricular zone emerges around E13 and, by E16.5, it swelles to represent a very substantial contribution to the neurogenesis in the developing cortex. Concomitantly, the VZ gradually shrinks and, by the end of prenatal development, it contains only a single layer of ependymal cells.

3.2.4. Other dorsal telencephalic progenitor cell types

Short Neural Precursors (SNPs) receive their name due to their unique morphological features, and can be considered a slightly different population of RGCs, that have lost their basal attachment to the pia but retain their apical contact with the ventricular surface (Gal et al., 2006). Although they express Pax6 and not Tbr2, and are located in the ventricular zone, this progenitor cells have been

shown to be, both in terms of i) neurogenic properties –unlike RGCs, they are likely to directly generate neurons from the VZ (Stancik et al., 2010)- and ii) responsiveness to niche-derived cues like Notch downstream signaling (Mizutani et al., 2007), closer to IPCs and could represent a population of RG-derived cells undergoing conversion into IPCs.

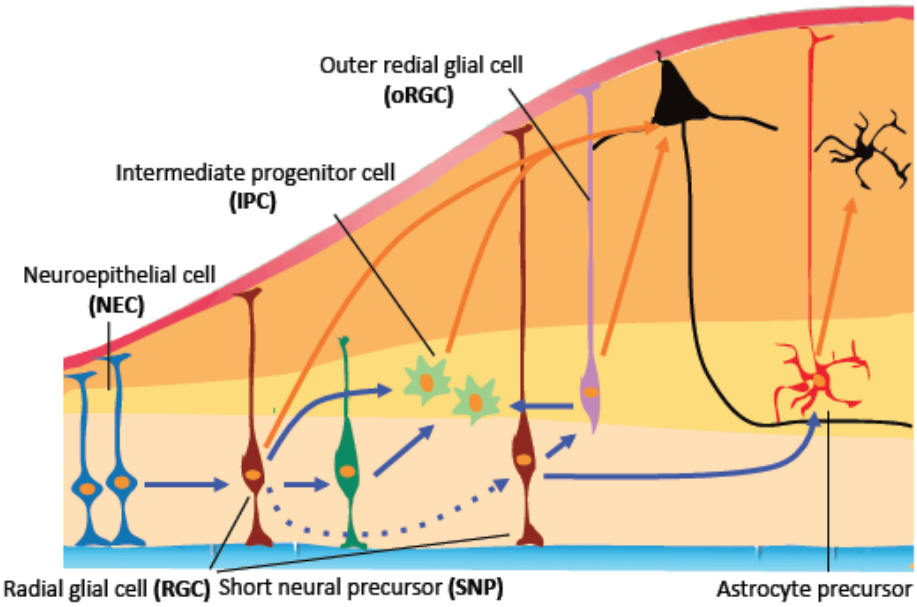


Figure 7. **Main types of cortical progenitor cells**.

Outer (Subventricular/basal) Radial Glial Cells (oRGCs) are abundant in human and other primates' developing cortex (Hansen et al., 2010). For long, they were thought to be present only in gyrencephalic mammals, but they have recently been shown to be also present in the developing lissencephalic murine cortex (Shitamukai et al., 2011). Morphologically, they are very similar to RGCs but, having lost their apical connection to the ventricular wall, are basally displaced to the outer (basal) edge of the SVZ. They retain their process towards the basal lamina, also express Pax6 and are capable to self-renew and generate intermediate progenitors, neurons and glia. These cells are thought to be responsible for the enormous tangential expansion of the neocortex in primates (Reillo et al., 2011).

4. Molecular control of cortical progenitor cell proliferation and neurogenesis

Cortical progenitor cell proliferation is a very tightly regulated process. Studies in *Drosophila* firstly demonstrated that the spindle plane orientation during neural precursor cell division determines the homogeneous (**symmetric**) *versus* heterogeneous (**asymmetric**) inheritance of the cytoplasmic content (Siller and Doe, 2009). In vertebrates, the outcome of the radial glial cell divisions is also greatly affected by the mitotic spindle orientation and, therefore, alterations in the regulation of the

cellular machinery governing this process have a great impact on the balance between self-renewal and generation of neurons and IPCs (Sanada and Tsai, 2005). The nature of neural stem cell divisions, i.e., whether they are self-renewing, indirectly neurogenic –via the generation of an IPC- or directly neurogenic, is controlled by a variety of transcriptional master-regulators, some of which respond to extracellular cues to orchestrate the precise timing of generation of the diversity of pyramidal neurons and macroglia (Qian et al., 2000).

4.1. Cell-autonomous factors

Many transcription factors have been implicated in the regulation of cortical progenitor cell proliferation and, thus, in the number of neurons generated during corticogenesis. Among them, and for the sake of simplicity, only the homeodomain-containing **Pax6**, basic-helix-loop-helix (**bHLH**) domain-containing TFs and the T-box-containing **Tbr2** will be discussed in more detail (see Fig 8).

4.1.1. Pax6

Pax6 is expressed by early neuroepithelial neural stem cells (Walther and Gruss, 1991), as well as other developing –and adult- tissues. Then, its expression is inherited by radial glial cells, in which it master-regulates the transcription factor network that controls neurogenesis. Pax6 is a highly conserved transcription factor containing two DNA binding domains, a paired domain (**PD**) and a paired-type homeodomain (**HD**). Pax6 plays a key role defining **dorsal identity**, so studies about the role of Pax6 in the regulation of proliferation and neurogenesis in RGCs have classically been affected by the fact that loss of Pax6 function is associated with the ventral identity colonization of dorsal territories (Quinn et al., 2007). Its mutation leads to **anyridia** in humans (Ton et al., 1991), and to the "*small eye* (**Sye**) " phenotype in mice and rats (Hill et al., 1991), very similar to the Pax6 knockout in a single allele, with no gross effect in brain structures. Homozygous mutation or knockout of Pax6 leads to death soon after birth, and to the failure to generate eyes, nose and neocortical structures. Pax6 acts in neural stem cell self-renewal and neurogenesis by tuning the expression of several kinds of genes, including transcription factors like **Hmga2**, Neurogenin2 (**Ngn2**) and **Tbr2/Eomes**–positively regulated by Pax6- and **Mash1/Ascl1** as well as Pax6 itself –negatively regulated- (see Fig.

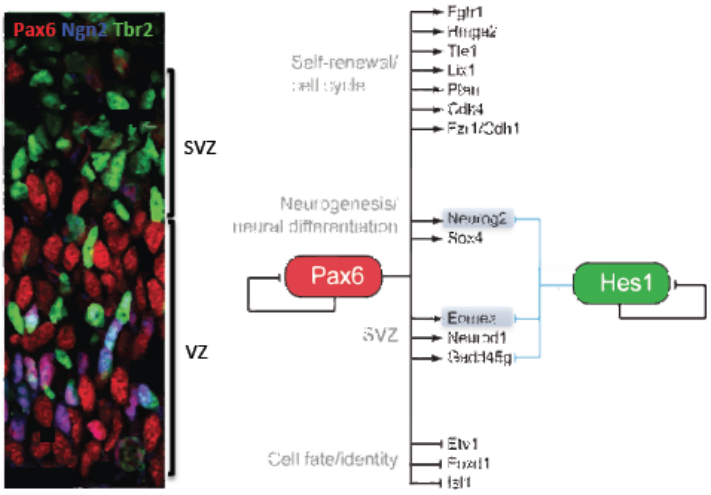


Figure 8: **Key transcription factors controlling pyramidal precursor proliferation and neurogenesis.** *A) Micrograph showing the expression pattern of the Paired-box-containing Pax6, the basic-Helix-Loop-Helix-containing Ngn2 and the T-box-containing Tbr2 transcription factors in the early embryonic cortical VZ and SVZ, reproduced from (Kowalczyk et al., 2009). B) Detail of the transcriptional regulation program governed by Pax6 in dorsal telencephalic progenitor cells. Note the antagonic roles of Pax6 and Hes1 in the expression of the proneural genes Ngn2 and Tbr2*

8), signaling molecules like **Fabp7**, cell-cycle regulators like **Cdk4** and **p27^{kip}** , and adhesion molecules like **L1**, some **cadherins** and **integrins** (Osumi et al., 2008; Sansom et al., 2009). Both gain and loss of Pax6 function result in a premature progenitor cell depletion, by excessively driving IPC generation or misexpression of ventral progenitor specification genes, respectively. Recent evidence suggests that Pax6 regulates proliferation, neurogenesis and dorsal identity through distinct subdomains (Walcher et al., 2013). Overall, a given radial glial cell behavior is crucially dependent of Pax6 protein levels, which are tightly regulated by a variety of mechanisms other than the transcriptional regulation, including the E3 ligase **Trim11**-dependent proteasomal degradation (Tuoc and Stoykova, 2008b).

4.1.2. bHLH factors

Transcription factors with **bHLH** motifs modulate critical events in during mammalian neocortex development. They are central regulators of cortical progenitor cell balance between proliferation and neurogenesis. This family of transcription factors include both pro-proliferative regulators, such as the bHLH factors from the **Id** and **Hes** families and the proneural genes **Ngn1** and **Ngn2** and **Mash1/Ascl1**, all playing important roles during cortical development (Martynoga et al., 2012). The proliferation-to-neurogenesis switch involves a coordinated increase in the activity of proneural bHLH factors and a decrease in the activity of Hes and Id factors (Ross et al., 2003). Although they are not absolutely required for RGC identity, loss of both Mash1 and Ngn2 proneural bHLH factors leads to the disorganization of the radial glia and to the inhibition of neuronal generation; instead, neural precursors either maintain a multipotent status or prematurely generate astrocytes (Nieto et al., 2001). Ngn2 expression is directly promoted by Pax6 (Sansom et al., 2009) and, at early corticogenic stages, Ngn2 is known to increase Tbr2 expression, thereby promoting intermediate progenitor cell identity (Schuurmans et al., 2004). However, later in development, Ngn2 is thought to act directly repressing Mash1 to prevent premature VZ proliferation exhaustion (Britz et al., 2006).
At the end of prenatal development, inhibition of proneural bHLH factors in cortical progenitors is required for the generation of astrocytes. Finally, the formation of oligodendrocytes is triggered by an increase in the activity of bHLH factors **Olig1** and **Olig2** (Ross et al., 2003).

4.1.3. Tbr2

T-box brain 2/Eomes is a transcription factor central for the regulation of indirect neurogenesis, through the transient amplifying progenitor cell intermediary, during neocortical development. It has been shown to be present in most (but not all) IPCs and, transiently, at least in some newborn pyramidal cells (Bulfone et al., 1999; Englund et al., 2005). Tbr2 cell-autonomously regulates the neurogenesis from intermediate progenitor cells: in the absence of Tbr2, cortical thickness is reduced as a consequence of impaired amplification of the neurogenic potential of cortical

progenitors by IPCs, and missexpression of Tbr2 by RGCs prematurely directs the apical-to-basal progenitor cell transition (Sessa et al., 2008). Although some reports defended that Tbr2 was solely required for upper layer pyramidal neuron production (Tarabykin et al., 2001), new evidences point to the contribution of Tbr2-driven neurogenesis to all cortical layers (Kowalczyk et al., 2009; Sessa et al., 2008). Also, Tbr2 is thought to regulate the integration of cortical interneurons via the chemokine Cxcl12 in a non cell-autonomous way (Sessa et al., 2010).

Noteworthy, the transcriptional regulation program that governs embryonic cortical progenitor proliferation and neurogenesis is similarly recapitulated in the adult neurogenic niches by neural stem cells and their increasingly differentiated progeny (see section 8).

4.2. Extrinsic factors

An array of extracellular signaling molecules, coming both from the progenitor pool itself or from other cellular entities, such as the neighboring IPCs and postmitotic pyramidal neurons and even further sources like meninges and CSF, have been shown to tune the activity and expression of these and other TFs expressed by cortical precursors and, consequently, the balance between neuron generation and neural stem cell self-renewal (Temple, 2001), Fig. 9. Among the signals originating in the progenitor compartment, one of the main players are **Notch** ligands, which act in a juxtacrine manner. Notch signaling represses proneural genes, like the bHLH Ngn2 and Mash1, and is important for the maintenance and self-renewal of RGCs during neurogenic stages of neocortical development (Mizutani and Saito, 2005). The onset of neurogenesis and the transition from NEC to RGC coincides with the onset of Notch signaling in the dorsal telencephalon, as detected by the expression of the major Notch ligand Delta-like 1 (**Dll1**) and the downstream transcription factors **Hes1** and **Hes5** (Hatakeyama et al., 2004). In the canonical Notch signaling pathway, Notch ligands binding to the transmembrane protein Notch promote the cleavage and release of the Notch intracellular domain, which then translocates into the nucleus to form a complex with **Rbpj**. This complex directly promotes the expression of the transcription factors Hes1 and Hes5 which, in turn, represses proneural genes (Mizutani et al., 2007). Polarization of apical complex proteins like **Numb/Numb-like** and **mPar3** result in one daughter cell with high Notch signaling that continues as a self-renewing RGC and a the daughter cell with low Notch signaling hat adopts an IPC or neuronal fate (Bultje et al., 2009).

In culture, growth factors like **BMP**, **FGF** and **EGF** family members have been shown to act in a concerted fashion to regulate proliferation and self-renewal of developing and adult neural stem cells (Lillien and Raphael, 2000; Temple, 2001). The identification of the sources for these and other ligands able to modify the behavior of the neural stem cells during corticogenesis provides important notions about how the developing tissue coordinates the timing of neurogenesis. Dll1 is exposed in

the surface of IPCs to help maintain the undifferentiated status in the radial glia (Nelson et al., 2013). **Retinoic acid**, secreted from the **meninges** and acting in NECs and RGCs through their pial endfeet, regulates cell cycle length and symmetric *vs* asymmetric cell division mode (Siegenthaler et al., 2009). Newborn pyramidal neurons secrete diffusible molecules, like the neurotrofin **Nt3** and proteins from the FGF family which, acting in a paracrine manner, help regulate the proliferation *vs* neurogenesis decision on the remaining progenitor cells, thus influencing the rate of neurogenesis and the lower-to-upper layer pyramidal neuron and neuronal-to-glial generation switches, respectively, in a process governed by the transcription factor **Sip1** in neurons (Seuntjens et al., 2009). Other neurotrophins like BDNF have also been shown to be important in adult hippocampal neurogenesis, as deletion of its receptor, TrkB, alters the integration of newborn neurons in the hippocampal circuit, with deletereus impact in behavior (Bergami et al., 2008), expanding an attractive model to explain how postmitotic neuron-released neurotrofins, such as likely Ntf3 and BDNF act as signals from the postmitotic part of the developing to coordinate several aspects of the biology of the remaining precursors (Fig. 9).

5. Progenitor lineage commitment

5.1. Spatial specification of cortical progenitors: from the progenitor protomap to mature cortical areas

Neocortical progenitor cells receive positional instruction from morphogens beginning at E9.5. Briefly, fibroblast growth factor 8 family members (**FGF8**, **FGF17** and **FGF18**) are secreted by the anterior neural ridge (anlage of the commissural plate), thus rostromedially (Crossley and Martin, 1995; Maruoka et al., 1998). Caudomedially, **WNT** and bone morphogenetic protein (**BMP**) family members are secreted from the cortical hem (Grove et al., 1998). Laterally, the WNT antagonist secreted frizzled-related protein 2 (**SFRP2**), epidermal growth factor (**EGF**) family members, transforming growth factor (**TGF**) **α** and **FGF 7** are secreted from the antihem (Assimacopoulos et al., 2003), Fig 2. Noteworthy, of the mentioned morphogens, only FGF8 has been demonstrated to function as a true organizer of area identity (Guillemot and Zimmer, 2011), as reduced FGF8 expression in hypomorphic mutants causes caudal areas of the cortex to expand rostrally (Garel et al., 2003), whereas increasing FGF8 expression by *in utero* electroporation leads to the caudal expansion

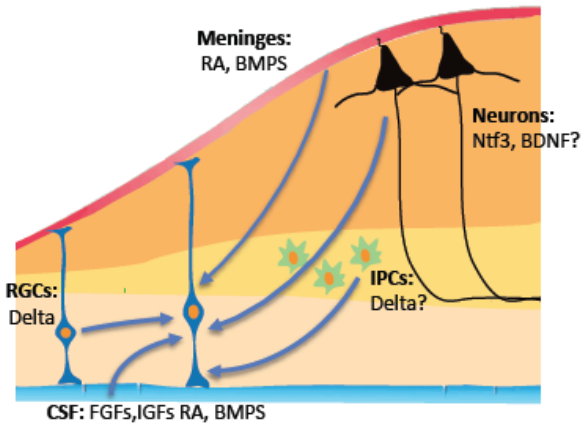


Figure 9. **Extrinsic factors regulating cortical progenitor cells self-renewal and neurogenesis**.

of rostral areas of the cortex (Fukuchi-Shimogori and Grove, 2001).

These and other diffusible molecules induce the gradient expression of transcription factors in the developing VZ progenitors that, in turn, delineate the area identity of their lineage. Four transcription factors, with complementary expression in the rostrocaudal and mediolateral axes are the main mediators of this patterning: paired box gene 6 (**Pax6**)(Walther and Gruss, 1991) (Bishop et al., 2000), empty spiracles homeobox 2 (**Emx2**) {Hamasaki, 2004 #324}, Chicken ovalbumin upstream promoter transcription factor 1 (**Couptf1**) (Armentano et al., 2007) and *trans*-acting transcription factor 8 (**Sp8**) (Sahara et al., 2007). Pax6 and Sp8 are involved in the acquisition of a **motor identity**, whereas Couptf1 and Emx2 promote **sensory area specification**. FGF8 family members promote rostral identity by repressing the expression of Emx2 and Couptf1, thus restricting their domains to the caudal regions (medial for Emx2 and lateral for Couptf1, Fig. 10. On the other hand, Sp8 and Fgf8 reciprocally promote each other's expression (Sahara et al., 2007) and this loop might cooperate in the promotion of rostral identity (O'Leary and Sahara, 2008). The orthogonal orientation of these four transcription factors' expression domains predicts than any given neuron is "predetermined" to acquire a certain area identity on the basis of the combination of transcription factors that it contains.

The protomap of the developing cortex's ventricular zone (Rakic, 1988), built by the morphogen-driven discrete domains of these four transcription factors' influence has then to be transmitted from radial glial cells to the intermediate progenitor pool, in a mechanism that seems to require the action of Tbr2 (Elsen et al., 2013)

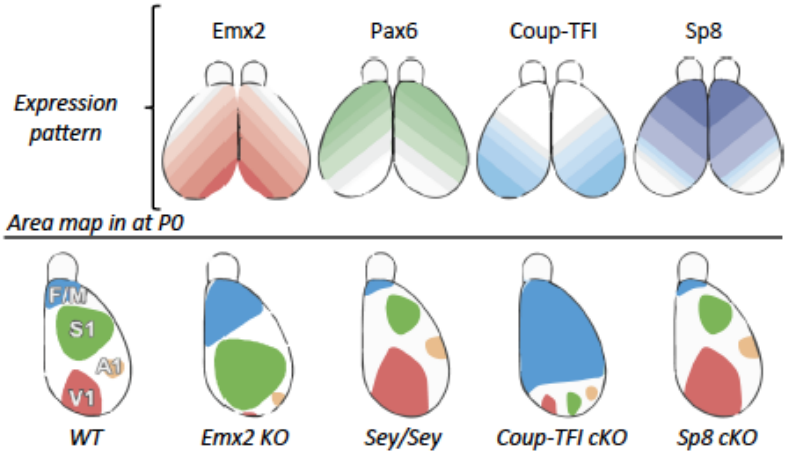


Figure 10. **Four transcription factors –Emx2, Pax6, Coup-TFI and Sp8- depict cortical progenitor area map**. *A) Illustration of the expression domains of the four TFs. B) Area map resulting from the genetic inactivation of these TFs at P0, clearly indicating their role in the promotion of the distinct area identities of the cerebral cortex. Modified from* (O'Leary and Sahara, 2008)

5.2. How is the timing of neurogenesis encoded? Two main theories. As mentioned before, cortical pyramidal neurons are born in a temporally-restricted manner: first layer I Cajal-Retzius cells, then deep-layer neurons and finally upper layer neurons. Therefore, mechanisms controlling the chronological modification of the newborn pyramidal cells' lineage commitment should exist. Cortical progenitors sequentially generate distinct subclasses of pyramidal cells that are partially fate-specified before exiting cell cycle. There are two main theories that might explain how this is

achieved: one stands that there is a **common cortical progenitor** cell, responsible for the generation of all the panoply of cortical pyramidal neurons (Fig 11, A). This theory stands that the exquisite order in which distinct pyramidal neuron lineages are born relies on the progressive loss of competence of the progenitor cell pool, i.e., as time elapses, a given progenitor cell loses the ability to generate early-born neurons and is only capable for giving later-born, upper layer pyramidal neurons. This theory is supported by a bulk of *in vivo* empiric data. For instance, retrovirus-mediated lineage-mapping experiments show that early-labeled clones generate a wide array of pyramidal lineages (Walsh and Cepko, 1988), while labeling clones later results in upper layer-only labeled neurons (Reid et al., 1995). Also, grafting experiments show that early stage progenitors introduced in an elder cortex are able to produce all kinds of pyramidal cells (McConnell and Kaznowski, 1991), whereas late stage precursors only generate upper layer neurons when grafted into younger cortices (Frantz and McConnell, 1996). Moreover, *in vitro* neuronal differentiation studies have also shown that neural and embryonic stem cells recapitulate the sequential generation of distinct pyramidal lineages found *in vivo* (Gaspard et al., 2008; Shen et al., 2006). The identification of the transcriptional regulators responsible for the progressive loss of the potential to generate all kinds of cortical pyramidal neuron lineages along corticogenesis is crucial for the confirmation of this hypothesis. For instance, **FoxG1**, a winged helix transcriptional repressor, has been identified as a fundamental player in the switch from Cajal-Retzius cell production to deep-layer neuron generation in the developing cortex (Hanashima et al., 2004), while factors driving the switch from deep to upper layer neuron generation remain unknown. In general, although these studies show that the overall capacity of the progenitor cell pool is progressively limited, they don't unequivocally demonstrate that the progenitor cell pool is homogeneous and evolves as a whole.

A second theory, raised more recently, defends that different subtypes of **lineage-committed cortical progenitors** coexist since the earliest stages of corticogenesis (Fig 11, B). The neurogenic

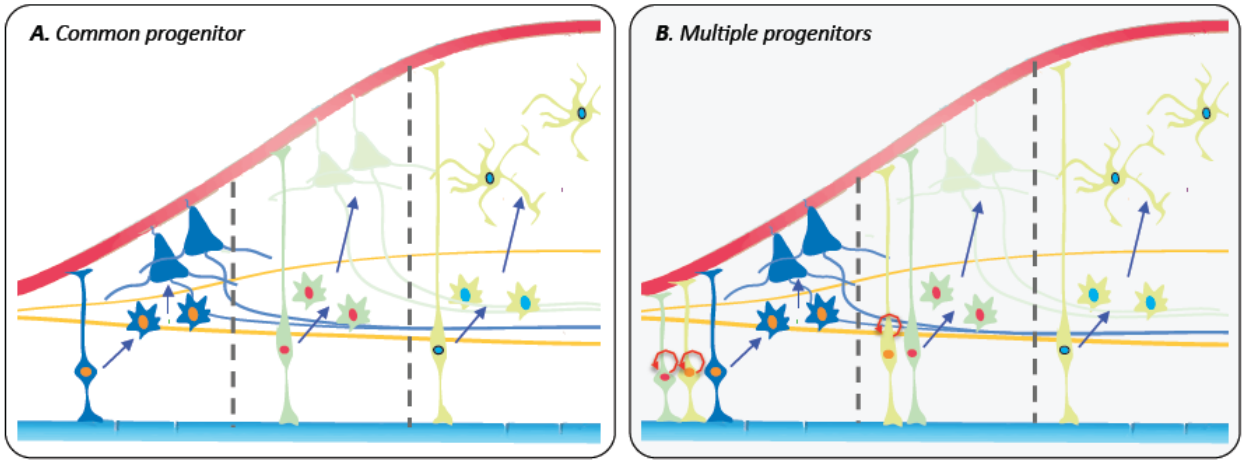


Figure 11. **2 models explaining how progenitor lineage commitment is achieved**. *Based on (Franco and Muller, 2013).*

capacity of each subtype is modulated in a way that permits the proper temporal scheme of cortical colonization by the different cortical pyramidal neuron subtypes. This notion was fostered by the discovery, around a decade ago, that some progenitor cells express markers previously known to identify certain pyramidal cell subtypes (Greig et al., 2013), such as Fez-family zinc finger 2 (**Fezf2**, formerly Fezl), labeling layer 5 subcortical projection neurons and also, in a scattered fashion, also some progenitor cells in the VZ (Chen et al., 2005; Molyneaux et al., 2005) or Cut-like proteins 1 and 2 (**Cux1** and **Cux2**), expressed by upper layer cortico-cortical projection neurons and also by some cells found in the VZ and SVZ (Nieto et al., 2004). Recently, an elegant study demonstrated that Cux2-positive radial glial cells are fated to generate only upper layer neurons, and explained how this progenitor subpopulation self-renew and expand in early stages of corticogenesis, getting ready to generate neurons when deep layers are already populated (Franco et al., 2012). In contrast, however, a more recent report (Guo et al., 2013), provides evidence against the existence of this Cux2-positive upper layer-commited progenitor subpopulation and, moreover, explains how lineage-traced Fezf2-positive RGCs sequentially generate deep layer neurons, upper layer neurons, astrocytes and oligodendrocytes, thus supporting that a common progenitor pool is responsible for the generation of the hole panoply of cortical pyramidal neurons. In summary, both theories explaining how pyramidal precursors are specified seem plausible and are supported by compelling empiric data. Hopefully, the advent of new genetic fate mapping approaches will cristalize in a deeper understanding of this fascinating process, likely bringing together part of the two theories apparently irreconcilable today. In any case, full pyramidal neuron specification is far for being fully achieved until postmitotic differentiation terminates (see section 7).

6. Migration of newborn pyramidal neurons

The newly generated pyramidal neurons then undergo radial migration towards their final destination in the neocortex. As previously introduced, the cerebral cortex is built in an inside-out fashion, so early-born pyramidal neurons establish in the prospective deep layers of the cortex, whereas later-born, upper layer neurons have to migrate greater distances to reach their destination. The first notion that newborn neurons employ radial fibers to radially migrate through the cortical thickness come from seminal studies by Cajal and, especially, Magini, who discovered nuclei intimately associated to glial varicosities "*along which they seem threaded like the grains of a rosary*" (Bentivoglio and Mazzarello, 1999). Later, the advent of new techniques, such as electron microscopy and **birth-dating** experiments, allowed the confirmation of these seminal observations and established the basis for today's knowledge about **glia-guided radial migration** (Angevine and Sidman, 1961; Rakic, 1972), Fig. 12. Radial glia-aided radial migration coexists with another mode of migration, **somal translocation**, prevalent in early stages of corticogenesis (Nadarajah et al., 2001).

The glia-independent migrating neurons are characterized by a long basal process towards the pial surface and a short trailing tail. Radial migration of projection neurons towards the CP does not follow a straight-forward route, as it includes phases of temporary migratory arrest and retrograde migration prior to entering the CP (Noctor et al., 2004), Fig. 12. Several human developmental neuropathologies have an origin in neuronal migration. The identification of the genetic basis of these pathologies, such a **lissencephaly**, **pachygyria** and **subcortical band heterotopia**, among others, usually characterized by drug-resistant epilepsy, has led to the identification of the genes implicated in the control of radial migration: cytoskeletal proteins like **TUBA1A**, **TUBB3** and cytoskeletal regulators as doublecortin (**Dcx**), **PAFAH1B1/LIS1** and **FilaminA** (Kwan et al., 2012; Liu, 2011; Manent et al., 2009) play a central role in this process. Also the proneural bHLH factors **Ngn2** and **Ascl1/Mash1** have been shown to play an important role in the regulation of radial migration through the modulation of the small G-proteins **Rnd2**- and **Rnd3**-mediated inhibition of **RhoA**, thus ultimately controlling the remodeling of the cytoskeleton (Heng et al., 2008; Pacary et al., 2011). Extracellular cues exert a prominent function in the control of radial migration. The most studied of them is Reelin. This glycoprotein is released by the Cajal-Retzius cells, transiently located in the marginal zone of the developing cortex (and also in the hippocampus), generating a gradient throughout the cortical wall in which the polarity of radially migrating neurons rely. After extracellular proteases-mediated cleavage, reelin acts through ApoER2 receptors in migrating neurons, promoting their progression beyond the older, already settled neurons. Reelin was identified by the analysis of the *reeler* mice that, carrying a homozygous mutation on the reelin gene, exhibit a completely disrupted cortical layering (Caviness, 1982).

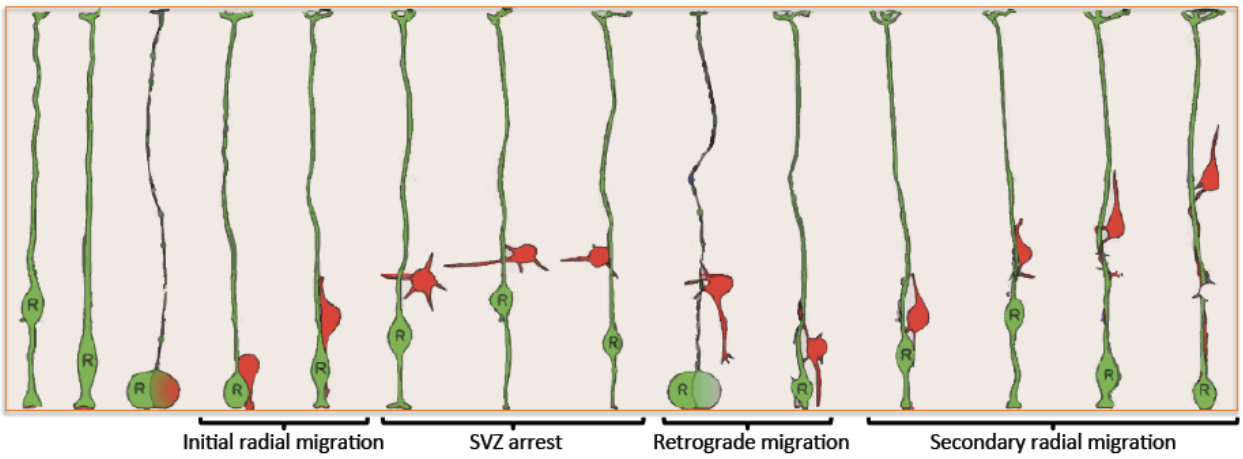


Figure 12. **Radial migration in the developing cortex.** *This process comprises different stages, that can be summarized, following the red cell in the illustration, aided in its radial migration by the radial glial scaffold: i) initial radial migration, ii) SVZ arrest, iii) retrograde migration, and iv) secondary radial migration. Adapted from* (Noctor et al., 2004).

7. Postmitotic pyramidal neuron specification

Concomitantly to cell cycle exit, a new set of transcription factors take over the control of pyramidal neuron specification in the cortex. Recent data coming from high throughput analyses of the differential transcriptomes in each projection neuron subtype has extraordinarily broaden the understanding of postmitotic cortical pyramidal neuron differentiation. The discovery of the selective expression, in each subset of cortical pyramidal neurons, of genes encoding different transcriptional regulators (Gray et al., 2004) has yielded a specific and non-overlapping map of transcription factors required for the postmitotic specification of the different cortical projection neuron types. Today we know that many of these transcriptional regulators are coexpressed at varying levels in the different pyramidal neuron subtypes, where they interact–reciprocally repressing each other's expression in many cases- to progressively refine the molecular identity and hodology in each pyramidal sublineage. In the next section, the main transcriptional regulatory programs necessary for the specification of corticofugal -corticothalamic (CTh) and subcerebral (SC)- and cortico-cortical - associative (A) and commissural/callosal (C)- projection neurons will be summarized. A **CThPN-> SCPN -> APN -> CPN** order will be followed – mirroring the inside-out pattern of colonization of the cerebral cortex by these neuronal populations during corticogenesis (Fig. 13).

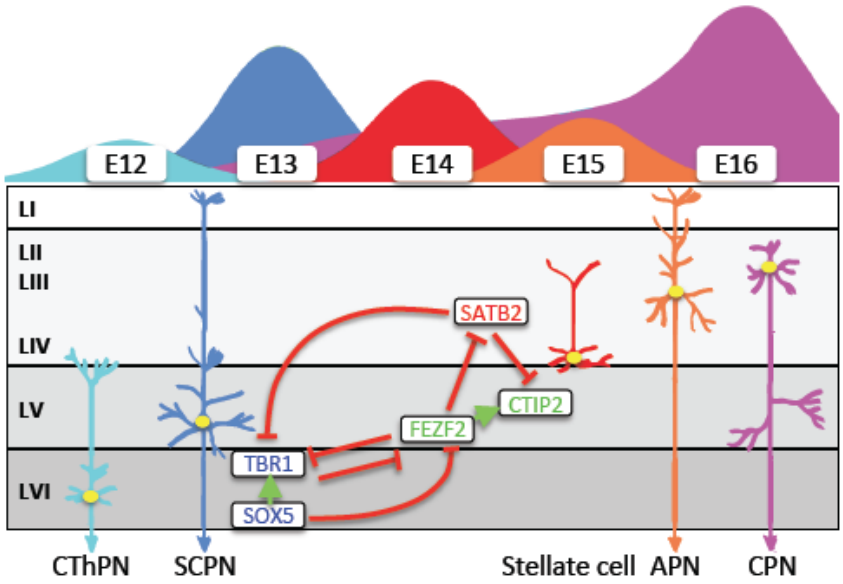


Figure 13. **Timing of the generation of the main of cortical excitatory neurons subclasses**. *Layer VI corticothalamic projection neurons (CThPNs) are born around E12; layer V subcerebral projection neurons (SCPNs), around E13; layer IV stellate cells, around E14; associative projection neurons (APNs) and commissural projection neurons (CPNs), most abundant in layers II/III are born around E15-16. Some important transcriptional regulators known to be important for pyramidal postmitotic specification, and their activation/repression relationships are also shown. Based on* (Franco and Muller, 2013).

7.1. Corticofugal PNs

7.1.1. CThPNs: **Corticothalamic PN** nuclei are mainly located in layer VI (Fig. 13). These neurons extend axons to specific thalamic nuclei: motor cortex CThPNs project to the ventral lateral and ventral anterior nuclei, somatosensory cortex CThPNs to the ventral posterior nucleus and visual cortex CThPNs to the lateral geniculate nucleus (Greig et al., 2013), Fig. 14. They are specified by a combination of transcription factors including the SRY-box containing gene 5 (**Sox5**) (Lai et al., 2008); the T-box brain protein 1 (**Tbr1**) (Bedogni et al., 2010) (McKenna et al., 2011) and a low dose of **Fezf2/Fezl** (McKenna et al., 2011), Fig. 13.

7.1.2. SCPNs: **Subcerebral PN** cell bodies are the largest in the cerebral cortex. They are preferentially located in cortical layer V (see Fig. 13). Attending to the projection phenotype, this group can be further subdivided into **corticotectal PNs**, that project to the superior colliculus –with collateral projections to the rostral pons- and whose nuclei are located in the visual cortex; **corticopontine PN**, that establish connections with the pons and **corticospinal PN**, that primarily project to the spinal cord, and can send collaterals to the striatum, red nucleus, caudal pons and medulla (Molyneaux et al., 2007), Fig. 14. The zinc-finger transcription factor **Fezf2/Fezl** has been demonstrated to be necessary for the generation and specification of subcortical projection neurons (Chen et al., 2005; Molyneaux et al., 2005). More recently, it has also been shown to be sufficient for the re-specification of postmitotic callosal PNs into subcortical PNs (De la Rossa et al., 2013; Rouaux and Arlotta, 2013). Downstream of Fezf2, CoupTF interacting protein 2 (**Ctip2**)/ **Bcl11b**, has also been shown to be necessary for the specification of corticospinal motor neurons (**CSMNs**), as mice lacking this gene fail to send axons to the spinal cord, showing important fasciculation aberrations (Arlotta et al., 2005) (Chen et al., 2005).

CThPNs and SCPNs share some features, like an early origin, a common gross connectivity pattern and the expression of many transcription factors, the relative abundance of which has been proposed to be the ultimate origin of their differential features. Indeed, a given CThPN can easily be re-specified to a SCPN by only switching on or off a single TF (Fezf2 and Tbr1, respectively), and vice-versa.

7.2. Cortico-cortical PNs

7.2.1. APNs: **associative projection neurons** are found spread in every cortical layer. They are either layer IV **stellate neurons**, which receive thalamic inputs and project within the same cortical column or **forward/backward PNs**, that send longer intrahemispheric projections, either to more rostral or to more caudal targets (Greig et al., 2013).

7.2.2. CPNs: **commissural/callosal projection neurons** nuclei are most abundant in layers II/III, but can also be found in LV and VI. CPNs extend projections towards the contralateral hemisphere through the *corpus callosum* or, alternatively, the anterior commissure (Molyneaux et al., 2009). Their postmitotic specification relies on a transcriptional regulation program that greatly functions by suppressing the SCPN program. The homeodomain-containing Special AT-rich sequence-binding protein 2 (**Satb2**), a well known transcriptional regulator known to preferentially bind to Matrix Attachment Regions (MARs) emerges as a key mediator of this transcriptional repression of the SCPN program: together with chromatin remodeling partners like the NURD complex (Britanova et al., 2008), Satb2 binds to the Ctip2 promoter region, repressing its expression and, therefore, CSMN specification. In the absence of Satb2, upper layer neurons are unable to cross the midline, begin to express CPN markers and extend their axons through the internal capsule (Alcamo et al., 2008). Other upper-layer neuron specific TFs, such as **Pou3F3/2** (formerly Brn1/2) and **Cux1/2** are also thought to play a role in CPN specification (Molyneaux et al., 2009).

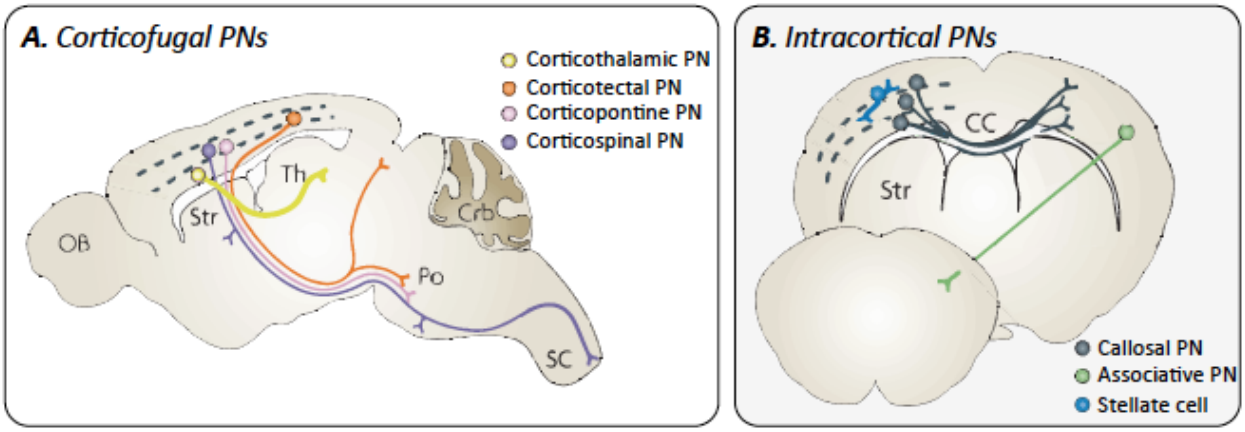


Figure 14. **Cortical pyramidal neurons hodology**. *A: Corticofugal projection neurons, their sommata located in the deep cortical layers V and VI, can be further subdivided into corticothalamic (CTh) PNs and subcerebral PNs (CSMNs, corticotectal PNs, corticopontine PNs). B, cortico-cortical pyramidal neurons are divided into associative PNs, callosal/commissural PNs and stellate cells. Modified from (Molyneaux et al., 2007).*

7.3. Extrinsic factors that regulate pyramidal neuron specification

An increasing bulk of data supports that the control over pyramidal neuron specification relies on the combinatorial effect of a series of cell-autonomous factors. However, during the postnatal critical period the sensorial inputs are highly required for the maintenance of the cellular identity of cortical neurons, as reflected by the fact that deprivation of sensorial information from either whiskers or eyes disrupts the columnar organization of the sensory areas of the cortex. Thereby, thalamocortical innervation, i.e., extrinsic factors, are also required to help establish and maintain cortical pyramidal neurons' phenotype (Li et al., 2013). A number of soluble factors have been identified that could play a role in cortical projection neuron specification, like BDNF (Fukumitsu et al., 2006), that has been shown to alter the laminar fate of pyramidal neurons, promoting a deep layer phenotype. Future

work is, nevertheless, required to build a comprehensive picture of the role of extrinsic factors in pyramidal neuron fate acquisition and refinement.

8. Adult neurogenesis

After corticogenesis is completed, some neurogenic niches of the developing brain evolve to maintain certain degree of neurogenic activity in the adult brain. The radial glial cells of the embryonic VZ/SVZ, after generating cortical astrocytes and oligodendrocytes, become astrocyte-like type B neural stem cells (Tramontin et al., 2003), (Merkle et al., 2004), Fig. 15.

Adult neurogenesis constitutes a unique form of structural plasticity. It has been best characterized in songbirds and rodents, but occurs also in other vertebrates, including humans (Kempermann, 2012). In most mammals, only two anatomically restricted neurogenic niches persist in the postnatal life, the subgranular zone (**SGZ**) of the hippocampal dentate gyrus (DG) and the lateral ventricles' subventricular/subependymal zone (**SVZ/SEZ**)(Zhao et al., 2008). Adult neurogenesis in both of these areas has been extensively characterized during the last decades, and the cell biology underlying the process, as well as many transcriptional determinants and extrinsic factors involved, have been identified. The functional relevance of adult neurogenesis is linked to the computational capacity of the neuronal network that specific new neurons integrate into: SVZ-derived adult newborn neurons normally migrate through the rostral migratory stream and integrate in the olfactory bulb network, where they become granular cells or periglomerular interneurons. This process is activity-triggered, as evidenced by the ability of different odorants to induce neurogenesis from this niche and the incomplete nature of the process in anosmic mice (Petreanu and Alvarez-Buylla, 2002). On the other hand, neurons generated in the SGZ then undergo a short migration and integrate in the DG as granular neurons. Recent evidence indicates an important role of adult-born neurons in cognitive and emotion-related hippocampal functions, such as pattern separation and anxiety-like behavior (Zhao et al., 2008).

Adult neurogenesis must occur in a balanced manner, as both too few and supernumerary newborn neural cells contribute to psychiatric disease. Reduced adult neurogenesis relates to reduced odor discrimination –essential for many species' survival-, accelerated cognitive decline, and mood alterations, such as major depression (Vadodaria and Gage, 2014). By the other side, epilepsy-driven excessive generation of new neural cells in the adult brain is also deleterious (Jessberger et al., 2007): it is unlikely to compensate for neurons lost due to seizures and some of its features, such as the abnormal morphogenesis, aberrant hilar migration and generation and sprouting of hilar dendrites (Walter et al., 2007) further worsen the disease. Besides the empiric approaches, the issue of adult neurogenesis' rate -specially in primates- has raised several theoretical questions. An

interesting one is the **stability-plasticity** dilemma: networks that are too stable are unable to learn, to acquire and store new information or to adapt to new circumstances and find novel solutions. On the other hand, too flexible networks are unable to long-lastingly store information, so they tend to forget prematurely. According to this concept, the incorporation of new elements (neurons and also glia) to the existing neural network has a dual impact on it: it represents the opportunity to increase the computational power of the system, while excessive neurogenesis would disrupt complex neuronal networks, such as the primate and human neocortex (Rakic, 1985). Interestingly, new computational models tell us that certain networks not only can cope with but even require new elements (neurons and glia again) to fulfill their capabilities (Kempermann, 2012).

The SEZ and the SGZ niches differ in several key aspects, mostly due to their differential ontogeny, but they also share some essential features, in terms of the spatial-temporal dynamics of the generation of newborn neurons (i.e., the sequential progression of the neural stem/progenitor cell-derived neuroblast to the mature neuron), and the cell-autonomous and extrinsic factors that govern the process (see section 4).

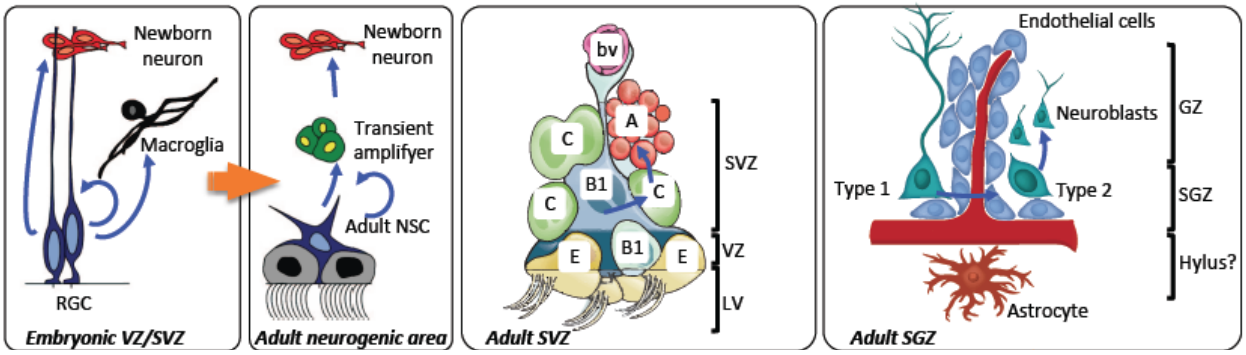


Figure 15. **Adult neurogenic niches in the brain.** *A, Illustration of the transition tom embryonic to postnatal neurogenic niches. B, C, the two main neurogenic niches in the adult brain areas. B, subependymal zone (SEZ), C, hippocampal dentate gyrus' subgranular zone (SGZ). The main cell types implicated are depicted. Adapted from* (Tramontin et al., 2003), *(Tong and Alvarez-Buylla, 2014; Vadodaria and Gage, 2014).*

9. The endocannabinoid system

The main aim of this doctoral thesis has been the elucidation of the mechanism of action and functional relevance of **endocannabinoid system (ECS)** signaling in the regulation of some important processes that take place during cerebral cortex development. In this brief introduction, the different elements of the system will be depicted, as well as the main processes it has been shown to control in the adult brain, where it has been more extensively studied. The implication of the ECS in the aspects of cortical development subject of study in the present work will also be summarized in section 10.

9.1. Historical perspective

Cannabis sativa (commonly known as marijuana) is a vascular plant known, used and domesticated by humans since ancient times. It was first discovered and used in the old Chinese civilization, over 5000 years ago, and, after that, its knowledge and use expanded to other cultures, both for practical reasons (its fibers have been and continue to be used as a primary source for the textile industry), for recreational purposes (still, it is the most universally used illicit drug in the world), in mystic rituals and, most importantly in the context of this Thesis, for medical purposes. Among the over 300,000 vascular plant species currently populating the Earth, *C. Sativa* is the only one known to produce the so-called cannabinoids. This molecules form a large family of compounds comprised by over 100 members so far, whose cyclic chemical structure shares a common feature: a high hydrophobic profile. As it will be discussed afterwards, this fact importantly affects its bioavailability and mode of action, as well as clearly distinguishes them from other drugs of abuse in terms of their pharmacokinetics. Among the many cannabinoids present in *C. Sativa*, there is a large consensus in the scientific community that **Δ^9-tetrahydrocannabinol** (**THC**) is the most important member, owing to its relative abundance and potency of action. This compound was first isolated in Raphael Mechoulam's laboratory in the 1960s (Gaoni & Mechoulam, 1964, (Mechoulam and Gaoni, 1965). It was almost another 30 years later when the gene encoding the protein responsible for recognizing THC and transducing its signal in THC-responsive cells was first cloned and characterized in the rat brain (Matsuda et al., 1990). Its human (Gerard et al., 1991) and mouse (Chakrabarti et al., 1995) ortologs were discovered only a bit afterwards. This protein was identified as the cannabinoid receptor 1 (**CB_1**), and only a few years later (in 1993), a gene encoding another cannabinoid receptor was identified in rat (Munro et al., 1993) and mouse (Shire et al., 1996) and named **CB_2**. Already in these seminal studies the differential distribution of the two proteins in the organism was recognized: CB_1 receptors are found in cells of the central nervous system, although they are also present in the periphery, and are the molecular mediators of most THC effects, including its

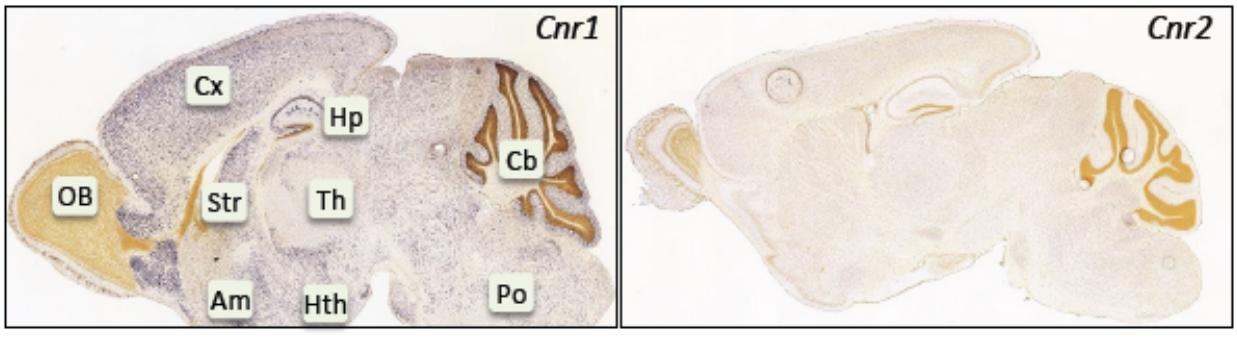


Figure 16. **CB_1 and CB_2 receptor mRNA distribution in the adult mouse brain**. *Note the profuse expression of CB_1 receptors throughout the different brain structures, whereas CB_2 expression in clearly weaker. Pictures taken from Allen Brain Atlas. Abbreviations: OB, olfactory bulb; Cx, cortex; Str, striatum; Am, amygdala; Hth, hypothalamus; Hp, hippocampus; Th, thalamus; Cb, cerebellum and Po, pons.*

psychoactivity. On the other hand, CB_2 receptor distribution is most prominent in non-neural cells, especially in the immune system, which underlies the immunomodulatory actions of cannabis, although some studies also point to the existence of some neuronal populations that express CB_2 (Van Sickle et al., 2005), see Fig. 16.

The CB_1 and CB_2 cannabinoid receptors share 44% sequence identity at the protein level, and are encoded by different genes. In both of them, the existence of various isoforms has been described. They belong to the G protein-coupled receptors (GPCR) superfamily. These receptors are, therefore, located to cellular membranes and composed of 7 transmembrane domains that leave 3 intracellular loops and 3 extracellular ones. Seeking for the molecular mechanism of some cannabinoid effects, which remained elusive, as pharmacological approaches had shown its occurrence independently of CB_1 and CB_2 receptor function, other proteins have been shown to have the potential to specifically bind cannabinoid ligands. Some of them are also GPCRs, like the formerly orphan **GPR55**, that only shares 14% protein sequence with CB_1 and CB_2 (Ross, 2009). Although lisophosphatidylinositol (LPI) has been shown to be the best endogenous agonist for this receptor, there is a bulk of pharmacological studies claiming that, at least in vitro, also some endocannabinoids like AEA and PEA, the phytocannabinoid cannabidiol (**CBD**) and some synthetic cannabinoid drugs like rimonabant/SR141716 and AM251, both inverse agonists of the CB_1 receptor, as well as the synthetic cannabinoid agonist CP55940, behave as GPR55 agonists (Kapur et al., 2009; Ryberg et al., 2007). Whether or not GPR55 is also an endocannabinoid receptor in physiological conditions is still a matter of debate. Other receptors have been ascribed a role in mediating cannabinoid responses: the transient receptor potential vanilloid receptor type 1 (**TRPV1**), a ligand-gated ion channel responsible for the transduction of the heat sensation upon capsaicin exposure, that binds arachidonic acid and has been also shown to be targeted by anandamide. Among other cannabinoid signaling-related functions, TRPV1 plays an important role in anandamide-dependent postsynaptic neuromodulation (Chavez et al., 2010; Grueter et al., 2010), see section 9.5. Noteworthy, some nuclear receptors belonging to the peroxisome proliferator activated receptor (**PPAR**) exert certain degree of cannabinoid ligand responsiveness, at least in certain cellular contexts.

9.2. Endocannabinoids

It is now universally accepted that cannabinoid receptors have not been evolutionary selected in order to bind phytocannabinoids. Instead, they are physiologically targeted by endogenous ligands, the so-called endocannabinoids (eCBs). These compounds, although strongly differing in their chemical structure, share the hydrophobic profile and the tridimensional position of their pharmacophores with THC (Pertwee et al., 2010).

9.2.1. Similarities and differences on the functional recruitment of the main endocannabinoids

The first molecule identified as an endogenous cannabinoid ligand was the amide of arachidonic acid and etanolamine (Devane et al., 1992), also known as anandamide, from the sanskrit *ananda* (meaning bliss) and commonly shortened as **AEA**. A short time later, another endocannabinoid was discovered, 2-arachidonoylglicerol (**2-AG**) (Mechoulam et al., 1995). Although the list of endogenous molecules known to be capable of binding and modulating cannabinoid receptors has grown notably in the last years (Pertwee et al., 2010), AEA and 2-AG are the best known eCBs, and are considered the most important ones attending to their relative abundance and regulatory role in a plethora of biological functions. Nowadays there is a growing consensus in the sense that 2-AG probably represents the most suitable eCB ligand for presynaptic CB_1, at least in most central synapses (Katona and Freund, 2008) while AEA, despite also acting through presynaptic CB_1 at some locations, under certain physiological conditions and, probably, in a sex-dependent manner (Huang and Woolley, 2012), could more likely be involved in complementary forms of endocannabinoid-mediated plasticity involving TRPV1 (Grueter et al., 2010; Puente et al., 2011).

Endocannabinoids are signaling molecules characterized by a common feature, that also distinguishes them from the vast majority of neurotransmitters: they are synthetized "on demand" from lipidic moieties, namely polyunsaturated fatty acid precursors, located in the cellular membranes.

9.2.2. eCB metabolism

Synthesis of eCBs involves the participation of specific enzymes, some of them well characterized nowadays (Di Marzo et al., 1994). The main biosynthetic pathway for AEA involves the participation of N-arachidonoylphosphatidylethanolamine phospholipase D (**NAPE-PLD**), that catalyzes the hydrolysis of N-arachidonoyl ethanolamines to give rise to AEA (Di Marzo et al., 1994), Fig. 17. NAPE-PLD is a calcium-sensitive enzyme, so its activity is increased upon cell stimulation. 2-AG synthesis from diacylglicerol (DAG) is mediated by diacylglicerol lipase (**DAGL**), another activity-sensitive enzyme of which two major isoforms, **α** and **β**, have been identified (Bisogno et al., 2003). Recent evidence points to the α isoform as the predominant in 2-AG synthesis in the brain (Gao et al., 2010; Tanimura et al., 2010) Fig. 17. DAGLα is known to be tightly regulated by its subcellular localization: its attachment to the scaffold protein **Homer** constitutes the molecular substrate for the functional coupling of the class I metabotropic glutamate (**mGluR**) receptors, also bound to Homer and coupled to phospholipase Cβ (**PLCβ**) activity, and endocannabonoid **retrograde signaling** (see Figs. 18, 19). This molecular arrangement channels the mGluR1/5 activity-dependent synthesis of DAG to its conversion into 2-AG (Jung et al., 2007), thus underlying eCB-mediated prevention of excessive

glutamate release and, therefore, endocannabinoid system function as a synaptic circuit breaker, with important pathophysiological implications (Katona and Freund, 2008).

The **inactivation** of eCBs is also tightly regulated, and it involves distinct enzymatic activities (Fig. 17): first, it is still a matter of debate whether the re-uptake of the eCBs from the synaptic cleft occurs by passive mechanisms or involves any kind of specific transporters. There is a body of compelling empiric data supporting the idea of a Na^+ gradient-independent membrane transporter for eCBs in neuronal and glial membranes, similar to that involved in other lipidic mediators (Piomelli, 2003). The fact that AEA uptake is saturable, substrate-specific and susceptible of inhibition by anandamide analogs and synthetic drugs (Beltramo et al., 1997; Hillard et al., 1997), points to the existence of such transporter. The recently identified catalytically-inactive form of FAAH –**FLAP**- (Fu et al., 2012) fulfills, at least in part, the features expected for such eCB transporter. In any case, whatever their pathway towards the cytoplasm is, the eCBs are then metabolized by specific intracellular serine hydrolases: Fatty Acid Amide Hydrolase (**FAAH**) is the main enzyme catalyzing anandamide breakdown to arachidonic acid and ethanolamine (Di Marzo et al., 1994) and monoacylglicerol lipase (**MGL**) is the main hydrolytic enzyme for 2-AG (Dinh et al., 2002), see Fig 17. The segregation of these enzymes in the two sides of the synaptic cleft (MGL is found mainly in the presynaptic button while FAAH localization is preferentially postsynaptic (Gulyas et al., 2004)) also suggests a functional segregation of the two main eCBs. Even though these enzymes are the best characterized ones, others contribute to the deactivation of endocannabinoids, including abhydrolases like **ABHD6** (Marrs et al., 2010), **COX2**, lipooxigenases and others (Ueda et al., 2013). Interestingly, eCB deactivation products are themselves the precursors for other lipid mediators with potent biological functions, such as **prostaglandins**, **prostamides** and **eicosanoids**, thus increasing the complexity of eCB metabolism, and even changing the outcome of endocannabinoid mobilization at least in some neurobiological contexts (Pazos et al., 2012). There are many other players involved in eCB metabolism (Ueda et al., 2013), but further explanations fall out of the scope of this work.

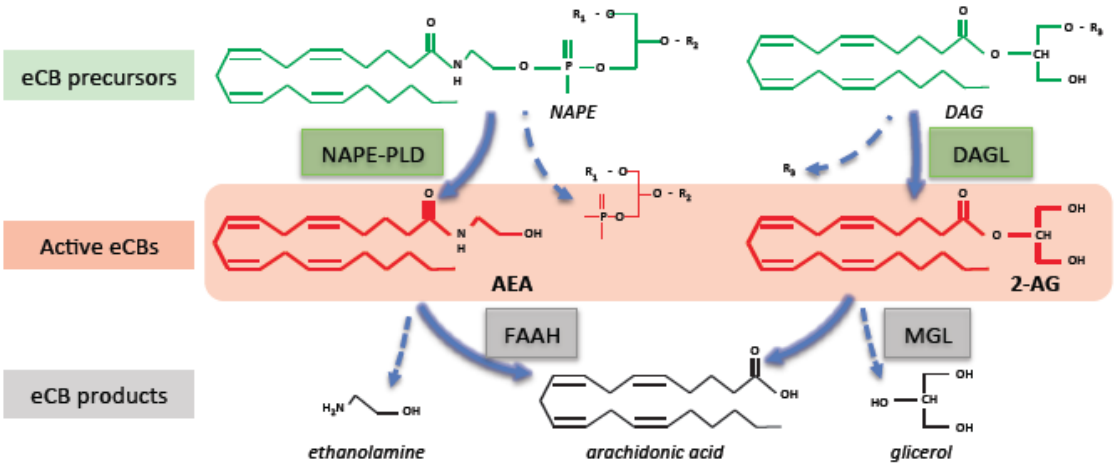


Figure 17: **Synthesis and degradation of AEA and 2-AG.** *Main metabolic intermediaries and enzymes implicated. Inspired in (Muccioli, 2010)*

9.3. CB_1 receptor structure and signaling

Cannabinoid signaling is diverse, and the outcome of cannabinoid receptor activation is critically dependent on contextual conditions such as, to mention a few, the cell type, the cellular (epi)genetic background, the nature of the ligand, the presence and activity of CB_1-modulating proteins, such as **CRIP1a** (Niehaus et al., 2007), posttranslational modifications of the receptor, such as **glycosylation** and **acylation** (Shim, 2010) and the interactions with other membrane receptors, for instance forming **homo- or heterodimers** (Mackie, 2005). As a GPCR, CB_1 tridimensional conformation oscillates between activated states –coupled to intracellular effectors- and non-activated ones. Binding of an agonist modifies the energetic equilibrium of the structure, causing the receptor to adopt a long-lasting shift toward the active steady-state. Due to the lipidic nature of the cannabinoid ligands, their binding site is atypically located in the hydrophobic region of the structure of these receptors, namely the transmembrane domain (Song and Bonner, 1996), this feature being shared by many, although not all, cannabinoid ligands, from an endogenous, synthetic and vegetal origin. Activation of CB_1 leads to the exchange of GDP for GTP in the α subunit of the **heterotrimeric G protein**. In neurons and other cell types, under physiological conditions, the G protein linked to CB_1 is usually inhibitory (**$G\alpha_{i/o}$**), as the cellular outcomes of the receptor's activity are generally blocked by pertussis toxin (Pertwee, 1997), so this is the signaling that is going to be summarized herein. Nevertheless, it is important to note that CB_1 can also be linked to other Gα subunits in some experimental paradigms, like Gq (Lauckner et al., 2005) and Gs (Hampson et al., 2000), though its physiological relevance is still a matter of debate. Back to the canonical CB_1 signaling, after the guanine nucleotide exchange, the $G_{i/o}$ α subunit inhibits adenylyl cyclase (**AC**) thus leading to the **decrease in the intracellular cAMP** concentration and, consequently, to the tempering of cAMP-dependent proteins' activity (protein kinase A (PKA) (Chevaleyre et al., 2007) and others, like Epac (Ramirez-Franco et al., 2014)). The **β/γ** subunits also plays a key role in the transduction of CB_1 activation: CB_1-dependent modulation of ionic conductance in the presynaptic terminal, including calcium channels closure (Kreitzer and Regehr, 2001) and potassium inward rectifying (**GIRK**) channels activation is clearly dependent on the β/γ subunits (Mackie et al., 1995). This ion channel activity modulation leads to the overall reduction of the membrane excitability in the presynaptic terminal, largely underlying short-term CB_1- mediated neurotransmitter release inhibition (Fig. 18). The β/γ subunits are also known to contribute to cannabinoid-induced activation of several intracellular signal transduction cascades, such as the **MAPK** routes (Howlett, 2005). Both the branch of the MAPK pathway regulated by cannabinoid receptor signaling and its sign (activation or inhibition) is highly cell context-, cannabinoid type- and dose-dependent: for instance, the canonical cannabinoid receptor-triggered extracellular signal-regulated kinase (**ERK)1/2** pathway activation, (Bouaboula et al., 1995; Galve-Roperh et al., 2002; Sanchez et al., 1998), section 10.1.2., can also be inhibited, as it has been reported in chronically cannabinoid-treated transformed cells (Galve-Roperh

et al., 2000) and in neural cells also treated with neurotrophins (Rueda et al., 2002). In other cell types, cannabinoid signaling is also able to modulate the **p38** (Derkinderen et al., 2001) and c-jun N-terminal kinase (**JNK)** pathways (Howlett, 2005). In neurons, the phosphatydil inositol 3-kinase (**PI3K**)-protein kinase B (**PKB**)/**Akt** - mammalian target of rapamycin complex 1 (**mTORC1**) pathway is also activated by CB_1 signaling, as shown in adult mice both under increased eCB tone (Busquets-Garcia et al., 2011) or THC administration (Puighermanal et al., 2009), see also section 10.1.2. This effect has been linked to the cognitive impairment caused by cannabinoid exposure. Therefore, in a mouse model of fragile X, a neuropathology characterized by overactive mTORC1 signaling, the therapeutic outcome of blocking CB_1 signaling with SR1 has been shown to be very similar to mTORC1 inhibition with temsirolimus (Busquets-Garcia et al., 2013). The cannabinoid receptor signaling-evoked modulation of the mTORC1 pathway is again the opposite in transformed cells, as it leads to the inhibition of mTORC1, which renders tumor cells susceptible to autophagy-driven apoptosis (Salazar et al., 2009).

Signaling by GPCRs, including CB_1, is typically terminated within seconds by mechanisms that include phosphorylation of intracellular residues, triggering decoupling from effectors (**desensitization**) and the recruitment of scaffolding proteins such as **β-arrestins** (Gainetdinov et al., 2004), consequently leading to CB_1 endocytosis (Nguyen et al., 2012). After elimination of bound ligands and dephosphorylation in endosomes, CB_1 can be either recycled back to the plasma membrane or degraded in lysosomes (Gaffuri et al., 2012).

9.4. CB_1 receptor localization

CB_1 receptors are profusely distributed throughout the mammalian adult CNS, indeed constituting one of the most abundant metabotropic receptors in the brain (see Fig. 16). CB_1 is expressed by different neuronal populations at varying levels: for instance, it is expressed at high levels in CCK-containing **GABAergic interneurons** while lower, though highly functional CB_1 expression is found in **glutamatergic projection neurons** (Marsicano and Lutz, 1999). The differential CB_1 expression levels required for its performance in these neuronal lineages has been shown to rely on the efficacy of its coupling to intracellular transducers (Steindel et al., 2013). CB_1 receptors are also expressed by **astrocytes** (Sanchez et al., 1998), in which they have been shown to play a key role in neuron-astrocyte communication (Navarrete and Araque, 2008), Fig. 19, and also in oligodendrocytes and microglia (Stella, 2004).

The subcellular localization of CB_1 receptors is diverse. They signal essentially from the plasma membrane, as they participate in paracrine cell communication. More accurately, CB_1 receptors are enriched in **presynaptic buttons**, from where they sense postsynaptically-released eCBs to inhibit neurotransmitter release, in GABAergic and glutamatergic neurons among others (Katona et al.,

1999; Katona et al., 2006) (Figs. 18, 19). There is also an intracellular pool of CB_1 receptors, localized mainly in endosomes, due to the constitutive endocytic cycle of the receptor (Leterrier et al., 2004) but also in other organelles. Among these "atypical" locations of CB_1 receptors, the **outer mitochondrial membrane** has recently emerged as a previously unexpected CB_1 location, where CB_1 receptor agonists have been reported to modulate the rate of mitochondrial respiration and, therefore, the energy availability in hippocampal neurons, playing a specific role in some CB_1-dependent neuromodulatory functions (Benard et al., 2012).

9.5. Physiological roles of the CB_1 receptor.

CB_1 receptor function has been most studied, by far, in the context of synaptic plasticity and nowadays we have access to detailed information on the mechanisms of CB_1-mediated forms of synaptic plasticity (Kano et al., 2009). The best characterized and probably more abundant mechanism of endocannabinoid-mediated modulation of synaptic function is **retrograde signaling**. It is a paracrine cellular communication, as –generally- endocannabinoids are released from the postsynaptic compartment in response to its activation and act through CB_1 – and maybe other- cannabinoid receptors in the presynaptic neuron (Fig 18). Different mechanisms govern short- and long-term forms of eCB-mediated synaptic plasticity. **Short-term depression** (**STD**) evoked by eCB signaling comprises two phenomena: "depolarization-induced suppression of inhibition" (**DSI**) (Ohno-Shosaku et al., 2001), in GABA-ergic terminals and "depolarization-induced suppression of excitation" (**DSE**) (Kreitzer and Regehr, 2001) in glutamatergic synapses. For the sake of simplicity, DSE will be used as a model: postsynaptic activity triggers [Ca^{2+}] increase in this compartment. Then, DAGLa, fed with DAG by PLCβ, which plays a coincidence detector role for postsynaptic activation

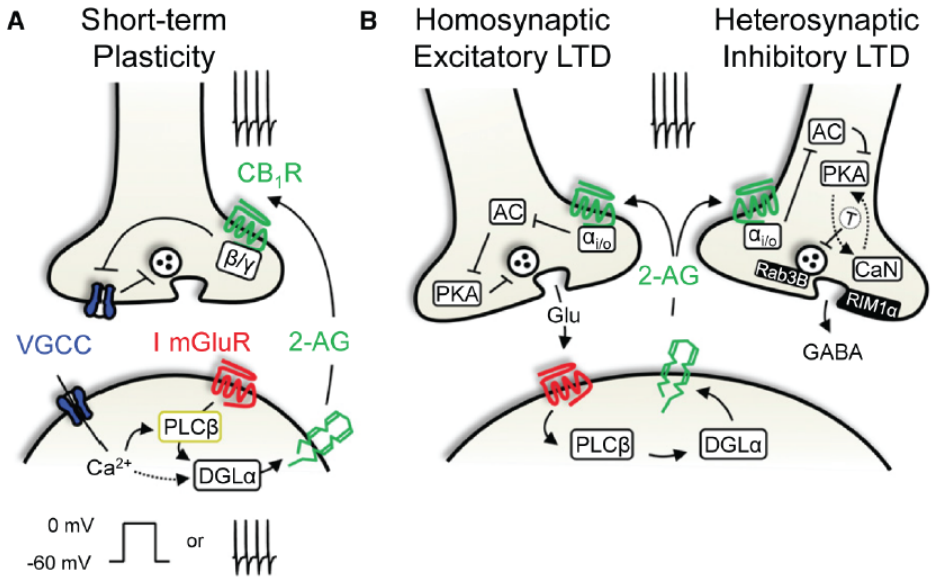


Figure 18: **eCB-mediated retrograde signaling**. *From* (Castillo, 2012).

(Ca^{2+}) and presynaptic input (glutamate acting on class I mGluRs), synthesizes 2-AG, which then travels backward to target presynaptic CB_1 receptors. The Gi β/γ subunit then inhibits calcium entry through the VGCCs' blockade, thus preventing more neurotransmitter to be released (Castillo et al., 2012). **Long-term depression** (**LTD**) mediated by the eCB system involves the inhibitory α subunit of the G protein associated to CB_1 which, via the AC inhibition, leads to the decrease in [cAMP] in the presynaptic compartment, what renders PKA inactive and thus inhibits neurotransmitter release from glutamatergic neurons (Fig. 18). The involvement of the Ca^{2+}-sensitive phosphatase calcineurin and the Rim1α-Rab3B has been shown to be essential for LTD to be expressed in GABAergic cells (iLTD)(Castillo et al., 2012; Tsetsenis et al., 2011).

Two other forms of eCB-mediated synaptic plasticity have recently been discovered (Fig. 19): there are evidences for an autocrine endocannabinoid loop, involving postsynaptically generated AEA acting onto postsynaptic TRPV1 (and, in some locations, probably also CB_1 receptors) (Castillo et al., 2012), and also another kind of cannabinoid-mediated regulation of synaptic transmission involves a third cellular component, the astrocytes (Navarrete and Araque, 2008): eCBs also act on astroglial CB_1 receptors to, indirectly via **gliotransmitter release**, induce plasticity at distant synapses (Navarrete and Araque, 2010).
There is a plethora of physiological and behavioral outcomes of eCB-mediated synaptic modulation (Fig 20). It plays a crucial role in the **extinction of aversive memories** (Marsicano et al., 2002), and closely interacts with **stress** signals (Senst and Bains, 2014), modulating one another. It is also

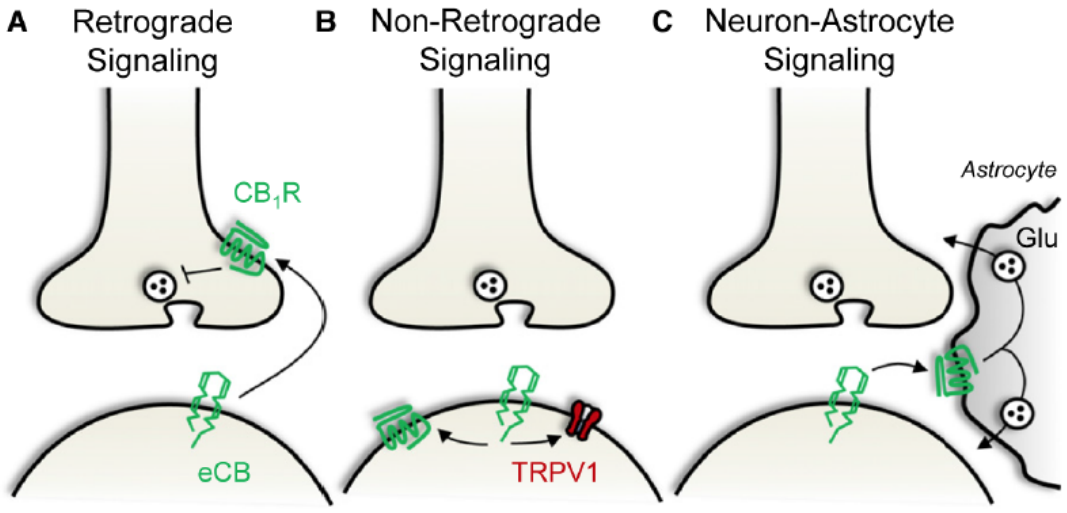


Figure 19. **Forms of eCB-dependent synaptic plasticity**. *From* (Castillo, 2012)

protective against **excitotoxicity** (Katona and Freund, 2008; Monory et al., 2006), participates in the regulation of **nociception** together with CB_2, both in the CNS and peripherally (Cravatt and Lichtman, 2004), controls **energy balance** (Quarta et al., 2010) and **feeding behavior** (Bellocchio et

al., 2010; Soria-Gomez et al., 2014), among many other functions. The pharmacological intervention over these CB_1-regulated processes represents a great therapeutic opportunity in the context of several human neuropsychiatric conditions, ranging from obesity to post-traumatic stress.

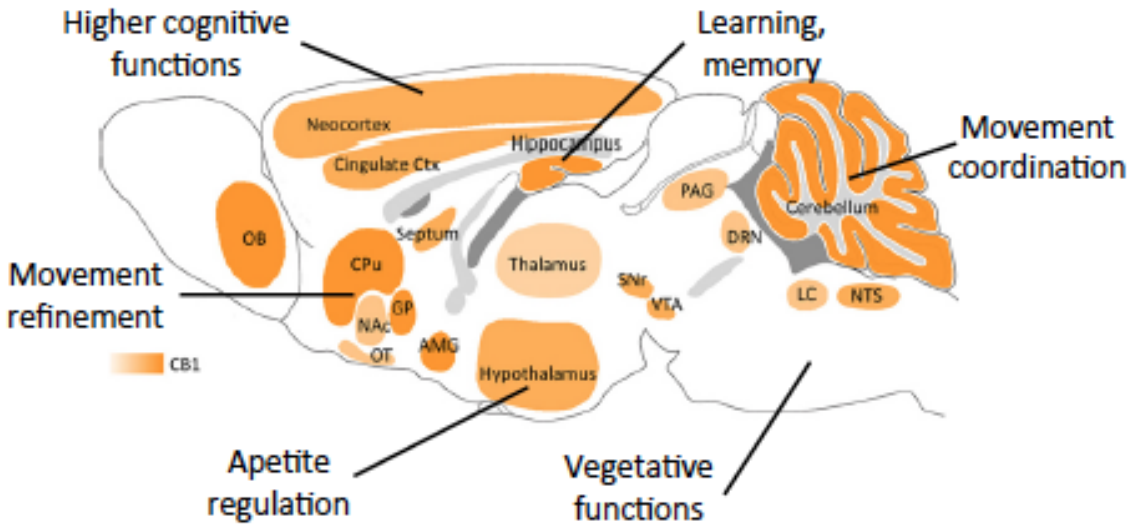


Figure 20. **Some key physiological functions of the eCB System**. *Adapted from (Flores et al., 2013).*

10. General aspects of the endocannabinoid system in CNS development

The eCBs, their major synthesizing and degrading enzymes and their specific receptors are present in mammalian organisms since very early steps in development. Indeed, the endocannabinoid system has been shown to play a role from the earliest ontogenic stages, i.e., periimplantation embryo development (Sun and Dey, 2008), when excessive endocannabinoid –mainly AEA- abundance or exacerbation of CB_1 activity by exogenous cannabinoid administration has been shown to delay embryonic development. Moving forward in development, and focusing in the CNS development, a role of the eCB system has been reported in neural plate formation (Psychoyos et al., 2008). In mammals, the levels of the main eCBs are detectable since the very earliest stages of development (Fernandez-Ruiz et al., 2000), being 2-AG generally orders of magnitude more abundant than AEA. Noteworthy, embryonic 2-AG levels are similar to the ones found in adult brain tissue, and they show a significant peak right after birth (Berrendero et al., 1999; Keimpema et al., 2010). The evolution of the expression of the enzymes responsible for the synthesis and degradation of the main endocannabinoids (section 9.2.2.) through development has also been addressed, with different degrees of success: the AEA degrading enzyme FAAH has been localized in RC2+ radial glial cells –in vitro- and in adult neural stem cells (Aguado et al., 2006), and the 2-AG synthesizing and degrading enzymes DAGL β and MGL have been shown to be present in the developing cortex (Keimpema et al., 2010; Mulder et al., 2008), including RGCs in the VZ, by E14.5. However, most efforts have been

devoted to localize the cannabinoid's molecular targets, namely the cannabinoid receptors, during CNS development: CB_1 receptor expression was first identified in several regions of the developing nervous system in early stages of brain development, including cerebellum, caudate-putamen, hippocampal anlage, cerebral cortex and brainstem (Berrendero et al., 1998). From the very initial observations, it was clear that the distribution of cannabinoid receptors during development strikingly differs from the observed in the adult brain: unlike in postnatal samples, the binding of both [3H]CP55940 and WIN55212-2-stimulated $^{[35S]}$GTPγS was found to be profusely located in the white matter –*corpus callosum*, anterior commissure, internal capsule…- (Fernandez-Ruiz et al., 2000; Romero et al., 1997). Interestingly, CB_1 mRNA was not present in these areas, suggesting that, instead of being expressed by non-neuronal cells in these locations, the cannabinoid receptor is localized to the developing axonal projections and plays a role in the process of axon guidance and target selection (Harkany et al., 2007). Interestingly, in support of this notion, early during postnatal stages, coinciding with the completion of the synaptogenesis period, CB_1 receptors' distribution is rearranged to the adult pattern (Berghuis et al., 2007; Morozov and Freund, 2003; Vitalis et al., 2008). In any case, the transient "atypical" localization of CB_1 receptors is highly suggestive of **development-specific roles of the cannabinoid receptor**. Noteworthy, similar observations have been reported in human developing brain samples (Mato et al., 2003). This seminal works fostered the emergence of a new research line, dedicated to the descriptive and mechanistic analysis of ECS functions during CNS development. New knowledge emerged since then will be summarized in the next section.

10.1. Role of the endocannabinoid function in neural progenitor proliferation

10.1.1. Expression of the cannabinoid receptors in neural stem/progenitor cells

While cannabinoids from different origins have been shown to modulate neural stem and progenitor cell proliferation, self-renewal and neurogenesis, both in the developing and in the adult mouse brain (Harkany et al., 2007), the expression levels of cannabinoid receptors by neural stem and progenitor cells is as yet a matter of debate. Although expression in cultured primary precursor cells (**neurospheres**) was proven some years ago (Aguado et al., 2005), technical drawbacks have kept *in vivo* CB_1 and CB_2 receptors' expression levels still to be fully elucidated. While some previous studies state that, both the mRNA by *in situ* hybridization, and at the protein by immunofluorescence detection means, CB_1 cannabinoid receptors are present at detectable levels in cortical progenitor cells *in vivo* (Mulder et al., 2008), they appear to be restricted to postmitotic pyramidal neurons, and thus under detection levels in the proliferative cells of the developing cortex in other studies (Vitalis et al., 2008). CB_2 receptor expression has been equally difficult to determine: by one side, the lack of reliable antibodies is a great inconvenient but, on the other hand, as CB_2 is assumed to be largely absent from postmitotic pyramidal neurons, both the mRNA and the protein expression levels

detected in the developing cortex are attributed to mitotic cells. Noteworthy, as stated in the previous section, "more is not always better", as for instance really low levels of CB_1 receptor expression in glutamatergic neurons have been shown to mediate many eCB-dependent processes and behaviors (Monory et al., 2007; Monory et al., 2006). So, even being scarce in neural progenitor (**NP**) cells, CB_1 receptors play an important role in the regulation of their biology.

10.1.2. Cannabinoid receptor signaling in neural stem/progenitor cells

An important question when approaching the study of cannabinoid receptor signaling in NPs is the origin of their ligands, before the structural substrate of the endocannabinoid communication in the mature brain (synapses) has been arranged (see section 9.2.2.). Noteworthy, it has to be taken into account that, as GPCRs in general, CB_1 receptors exhibit a tonic activity, due to the "spontaneous" transitions from active – coupled- to inactive –uncoupled- conformations and vice-versa (Pan et al., 1998) (section 9.3). Having said that, it has been proven not only that endocannabinoids are present in developing neural tissues (Berrendero et al., 1999), but also that both AEA and 2-AG can be released, upon challenge, by neural precursor cells (Aguado et al., 2005), thus providing a molecular logic for **auto- and paracrine eCB signaling in NS/PCs**. Generally, CB_1 "signaling modes" in NS/PCs can be classified in two main branches: i) the canonical Gi/o signaling of the cannabinoid receptor and ii) the result of the cooperation between CB_1 and other membrane receptors, including heteromerization of CB_1 receptors with other GPCRs, which has been proposed to take place in several cell types and is also likely to play a role in endocannabinoid signaling during development (Harkany et al., 2007), and also growth factor/neurotrophin receptors, generally tyrosine kinase activity-coupled receptors. Signaling from cannabinoid receptors is thereby highly dependent on the cell type, the physiological context and the membrane partners involved and, specially in the rapidly evolving context of the developing cortex, this has to be taken into account when considering the complexity of endocannabioid functions.

CB_1 cannabinoid receptor engagement has been proven to be positively coupled to developing and adult **NP cell proliferation** (Aguado et al., 2005; Jin et al., 2004; Mulder et al., 2008) and inhibition of neural differentiation (Rueda et al., 2002), both physiologically and under excitotoxic insults, like kainate exposure (Aguado et al., 2007). Therefore, CB_1 knockouts show impaired NP proliferation both in the developing VZ/SVZ and in the adult dentate gyrus and SEZ, whereas $FAAH^{-/-}$ mice, with increased endocannabinoid tone, showed the complementary phenotype (Aguado et al., 2005; Mulder et al., 2008). Also CB_2 cannabinoid receptor has been shown to promote developing and adult NP proliferation, both physiologically and in neurodegenerative conditions (Palazuelos et al., 2006). The cannabinoid receptors' signaling functions in neural stem and progenitor cells rely in the modulation of specific intracellular signal transduction pathways, including modulation of the

extracellular signal-regulated kinase (ERK) (Galve-Roperh et al., 2008), that has been shown to rely on various upstream events: i) CB_1- Gi-driven depletion of cAMP levels, and consequent inhibition of PKA, what de-inhibits ERK signaling cascade; ii) CB_1-associated G-protein β/γ subunits also stimulate ERK signaling in a PI3K-dependent manner and iii) ligand-independent transactivation of multiple tyrosine kinase family receptors for growth factors, such as the EGFR, IGF-1R, VEGFR, TrkB and possibly others, described in neuronal (Dalton and Howlett, 2012) (Berghuis et al., 2005) and astroglial (Galve-Roperh et al., 2002) cells and likely accounting for some of the CB_1-dependent functions in NPs. Also, the involvement of Src family kinases has been proven to be required in some cases (Berghuis et al., 2005; Galve-Roperh et al., 2002) (Dalton and Howlett, 2012). In addition, the PI3K/Akt axis, previously known to be modulated by CB_1 signaling in human astrocytoma cells (Galve-Roperh et al., 2002) and, together with mTORC1, more recently defined as a paramount mediator of the amnesic effects of anandamide (Busquets-Garcia et al., 2011) and delta-9 THC (Ozaita et al., 2007; Puighermanal et al., 2009) (section 9.3), has been shown to play a central role in CB_1-dependent modulation of NP proliferation of some origins (Trazzi et al., 2010), while not in others (Jiang et al., 2005), Fig. 21.

These and likely other signal transduction pathways converge to control the gene expression profile of these cells which, in turn, modulate multiple events of several cellular processes including, but not limited to, i) **cell cycle regulation**, ii) **progenitor cell identity determinants' expression** and iii) **control over cytoskeletal dynamics**. CB_1 signaling in proliferating cells has been proposed to involve the modulation of the expression and/or activity of certain transcription factors, for instance **CREB** (Soltys et al., 2010), **Stat3** (He et al., 2005; Zorina et al., 2010), **β-catenin** (Trazzi et al., 2010), **BRCA1** and **Pax6** (Bromberg et al., 2008), Fig. 21.

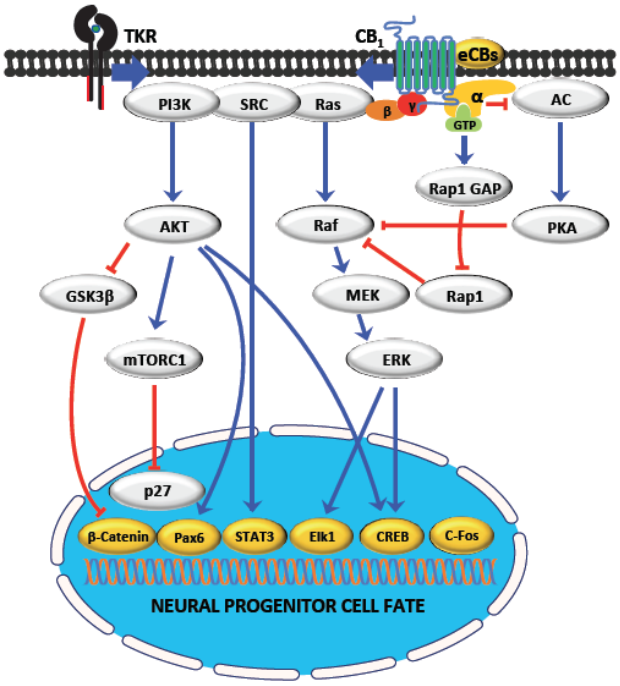


Figure 21. **CB_1 signaling in neural precursor cells**

10.2. Cannabinoid receptor signaling in postmitotic events of cortical development

CB_1 functions in neuronal differentiation have attracted the attention of many researchers, primarily because of the fact that, irrespective of the levels in mitotic cells, CB_1 expression clearly increases concomitant with neuronal differentiation (Begbie et al., 2004), what is suggestive of an important regulatory role for this receptor in postmitotic events of neural development:

10.2.1. Migration

The endocannabinoid system seems to play a facilitating role in neuronal migration in different contexts: in the fetal cortex, CB_1 receptors have been shown to **promote radial migration** of newborn pyramidal neurons, as both CB_1 agonists and eCB breakdown inhibitors increase the rate of migration (Mulder et al., 2008), Fig. 22. Also in the context of prenatal development, CB_1 is likely positively coupled to interneuronal migration, as altering CB_1 activity through embryonic THC administration (see section 10.3.) increases hippocampal colonization by cholecystokinin (CCK)-containing interneurons (Berghuis et al., 2005). However, other studies support that prenatal exposure to the synthetic mixed cannabinoid ligand WIN55,212-2 disrupts both radial and tangential migration {Saez, 2013 #680}, although the mechanisms involved and whether these treatments render CB_1 overactive or, alternatively, cause CB_1 desensitization are still open questions. In the postnatal brain, cannabinoid signaling has been reported to promote the migration of SVZ-derived neuroblasts towards the olfactory bulb (Oudin et al., 2011).

Promotion of neuronal migration by cannabinoid receptor relies, at least in part, on the modulation of specific cytoskeletal regulators: the modulation of RhoA activity is a likely candidate, as it has been shown participate in CB_1-guided neuronal morphogenesis (see next section, 10.2.2.) and recent evidence shows that cannabinoid signaling modulates migration of SVZ-derived neuroblasts along the RMS through the regulation of the strength of the interaction between actin crosslinking protein fascin interaction and the protein kinase C (PKC) (Sonego et al., 2013).

10.2.2. Morphogenesis

CB_1 signaling participates in various steps of neuronal morphogenesis: first, CB_1 activation **regulates neurite outgrowth** in cultured neuronal cells, although the outcome of such regulation varies in different studies: in some cases it has been shown to trigger neurite outgrowth (He et al., 2005; Jordan et al., 2005) whereas, in others, the opposite effect is reported (Vitalis et al., 2008)..Interestingly, retinoic acid-elicited neurite outgrowth in PC12 cells has been shown to rely on the increase of DAG lipases' expression (Jung et al., 2011), and thus in 2-AG signaling.

CB_1 is enriched in developing axons, both in the growth cone and in its most proximal axonal segment. In interneurons and retinal ganglion cells, activation of CB_1 has been shown to cause **growth cone collapse** (Argaw et al., 2011; Berghuis et al., 2007), suggesting that endocannabinoids act as chemotactic cues. In corticofugal axons (CFAs) a strikingly **altered fasciculation** is observed in CB_1-deficient embryos and early postnatal mice (Mulder et al., 2008; Wu et al., 2010). The disposition of the endocannabinoid synthesizing and degrading enzymes in the growth cone and initial axon segment has been studied in detail in these axons: CB_1 and DAGLα are expressed both in the growth cone and in the proximal axonal segment; MGL is absent from the growing tip, while abundant in the static axonal segment. This disposition supports the existence of an autocrine 2-AG

domain in the growth cone, that signals **axon elongation**. To prevent aberrant branching, MGL expression confines this 2-AG microdomain to the growth cone surroundings (Keimpema et al., 2010), Fig. 22. When axonal targeting is completed and synaptogenesis begins, MGL invades the growth cone –and apparently also the postsynaptic target-, thus terminating 2-AG- mediated axon elongation. The described CFA growth-promoting 2-AG microdomains could also be part of a paracrine communication: CB_1-containing CFAs meet CB_1-negative thalamocortical axons (TCAs) while traversing the external capsule. TCAs, in turn, express DAGLβ, what provides a molecular substrate for the tight association between these 2 types of axons, reciprocally instructing each other's navigation in the so-called "handshaking" model. Indeed, lack of CB_1 in CFAs not only affects their fasciculation, but also the TCAs' (Wu et al., 2010).

CB_1-mediated modulation of axonal guidance likely involves the modulation of cytoskeletal-regulatory proteins, such as **RhoA**, which activity is enhanced by CB_1 activation (Berghuis et al., 2007). A concerted action of Deleted in Colorrectal Cancer (**DCC**) and CB_1 has been reported during retinal ganglion cell axonal elongation, in virtue of which CB_1-mediated decrease in cAMP concentrations and, consequently PKA activity, is required for the appropriate targeting of retinothalamic axons, in a DCC-dependent manner (Argaw et al., 2011).

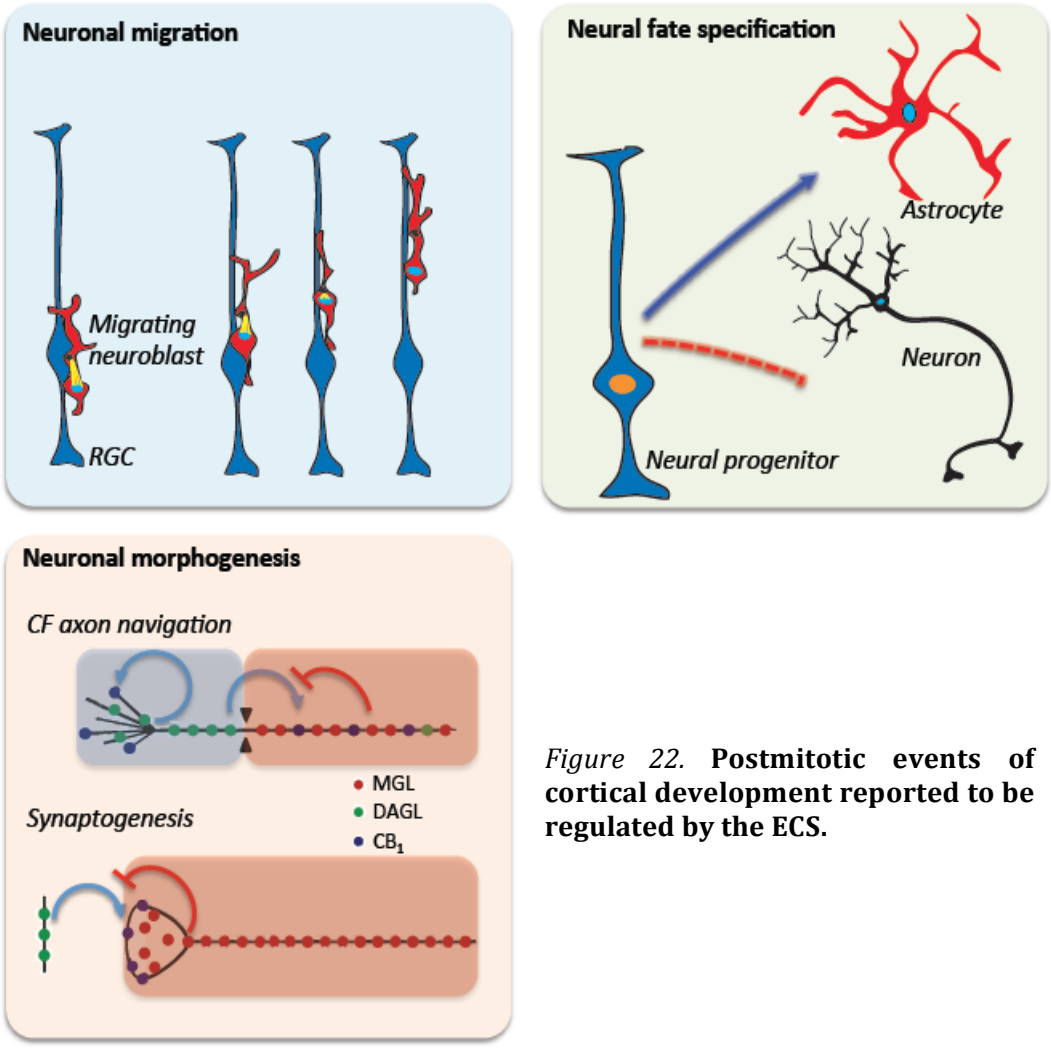


Figure 22. **Postmitotic events of cortical development reported to be regulated by the ECS.**

An interesting theory about CB_1 function in axon guidance involves the existence of a subcellular recruitment of CB_1 receptors, together with DCC and likely neurotrophin receptors to the lipid rafts abundant in the growth cone, therefore bringing together distinct intracellular transducers to signal growth induction or arrest (Keimpema et al., 2011).
Of note, also CB_2 receptors have been shown to participate in RGCs' axon guidance (Duff et al., 2013), widening the array of possibilities of eCB-medited tuning of axonal patterning.

10.2.3. Fate specification

The ECS participates in the delineation of the neural cell fate since the very earliest decisions. CB_1 signaling has been shown to modulate the neuronal *vs* glial nature of newborn neural cells. It has been reported that eCBs, acting through CB_1, **promote astroglial differentiation**, both in vitro and in vivo, through the induction of CREB (Soltys et al., 2010) and by enhancing the expression of astroglial markers, specifically by increasing the activity of the GFAP promoter (Aguado et al., 2006), Fig. 22. Interactions of the eCB system with the bHLH transcriptional regulators (section 4.1.2.) are also plausible, but require further investigation.
The instructive role of the eCB system in the delineation of specific neuronal and glial phenotypes has not been addressed in depth yet.

10.3. Developmental exposure to THC

Cannabis is, by far, the most commonly used illicit drug during pregnancy in the West countries. The consequences of prenatal exposure to cannabinoids have been addressed by many longitudinal human studies, as well as basic science research (Jutras-Aswad et al., 2009), encouraged by the need for a better understanding of the possible negative effects of cannabinoid exposure on the neuropsychiatric health of the offspring. About 1/3 of the THC blood content undergoes cross-placental transfer (Hurd et al., 2005), so it readily reaches the developing fetus' CNS. Many studies conclude that cannabinoid exposure during development sensitizes the CNS network to **cognitive impairments** (Huizink and Mulder, 2006), **drug abuse predisposition**, strikingly even in the subsequent generation (Szutorisz et al., 2014), and the onset of neuropsychiatric disorders, such as **schizophrenia** and **anxiety** (Jutras-Aswad et al., 2009). Recent work enables the proposal of a molecular network through which THC alters normal brain development to miss-wire the cerebral circuitry. This evidence points to: i) **epigenetic** modifications, specially in genes implicated in neurotransmission {Morris, 2011 #655}, ii) missexpression of specific proteins that regulate key developmental processes, such as **SCG10** (Tortoriello et al., 2014) and, iii) the alteration of the physiologically temporal- an spatially-confined endocannabinoid signaling, that play an important regulatory role in many aspects of cortical development (see section 10.2.) to an aberrant configuration (Keimpema et al., 2011). Likely, modifications of the endogenous cannabinoid signaling

elicited by developmental exposure to cannabis, either by overactivating cannabinoid receptors otherwise tempered in their signaling or by causing the desensitization and loss of function of normally active cannabinoid receptors underline the molecular, anatomical and behavioral consequences of embryonic cannabis exposure, still to be completely understood.

11. The endocannabinoid system in adult neurogenesis

Both CB_1 and CB_2 cannabinoid receptors have been ascribed a role in the regulation of **adult neurogenesis** (section 8). The initial reports of the modulatory role of endocannabinoid signaling in the regulation of adult neural stem/progenitor cells were rather controversial: an initial study reported that the endocannabinoid AEA inhibits adult neurogenesis (Rueda et al., 2002). Shortly after, it was shown that genetic deletion and pharmacological blockade of the CB_1 receptor resulted in an opposite variation of the number of proliferating cells, decreased in $CB_1^{-/-}$ *vs* WT but augmented in SR1- *vs* vehicle-treated mice, both in the adult SVZ and in the DG's SGZ (Jin et al., 2004). A more clarifying study came out little after, demonstrating the existence of a functional endocannabinoid system in adult NPs –capable of synthesizing and degrading endocannabinoids and to respond to them owing to the expression of CB_1. In this study, both the infusion of synthetic cannabinoids like WIN55212-2 and the enhancement of endocannabinoid availability via the genetic deletion or the pharmacological blockade of a major eCB-degrading enzyme –FAAH, section 9.2.2- result in an **increased adult NP proliferation**, in a CB_1-dependent manner, as evidenced by the ability of the CB_1 antagonist SR1 to block the aforementioned effects (Aguado et al., 2005). Besides controlling NP cell proliferation in basal conditions, CB_1-dependent regulation emerged as a key mediator of the excitotoxic insult-driven neurogenic response of the adult hippocampus, as shown in a more recent paper using kainate injections-induced epileptic seizures (Aguado et al., 2007). The molecular composition of the endocannabinoid loop responsible for the modulation of adult neurogenesis also includes 2-AG, as evidenced by the impaired adult neurogenesis found in $DAGL^{-/-}$ mice (Gao et al., 2010). Also, new studies point to the cooperation among FGF and EGF –key elements of the neurogenic niche, section 4.2- and endocannabinoid signaling to regulate adult NP cells' biology (Sutterlin et al., 2013).

The potential of the eCB system to stimulate adult neurogenesis has prompted the addressing of the potential therapeutic benefit of pharmacological interventions over the eCB system in neuropsychiatric disorders in which etiology adult neurogenesis is impaired, such as major depression (Jiang et al., 2005). Besides, the **anxiolytic effect** of the non-psychoactive phytocannabinoid cannabidiol (**CBD**) has been shown to rely on its **pro-neurogenic effect**, which requires the functionality of CB_1 receptors (Wolf et al., 2010).

Also CB_2 receptors, expressed in undifferentiated neural stem cells but largely absent from mature neurons, have been shown to be positively wired to adult NP proliferation in the two main adult neurogenic niches of the CNS (Goncalves et al., 2008; Palazuelos et al., 2006), both in basal conditions and under excitotoxic insults (Palazuelos et al., 2006). CB_2-mediated promotion of adult neurogenesis depends on 2-AG production and is enhanced by FAAH inhibition (Goncalves et al., 2008). CB_2 seems to act through the stimulation of the PI3K/Akt/mTORC1 intracellular signaling cascade to mediate its pro-neurogenic effects. Importantly, being CB_2 receptors devoid of the psychoactive effects of CB_1 stimulation, CB_2-selective agonists represent promising pharmacological tools for the stimulation of adult neurogenesis without the possibly undesired side-effects of promiscuous / mixed cannabinoid agonists.

2. AIMS OF THIS THESIS

Considering the available knowledge on the neurodevelopmental role of the endocannabinoid system we defined the following objectives for this Doctoral Thesis:

OBJECTIVE 1. To explore the role of CB_1 receptor signaling regulating dorsal telencephalic progenitor cell identity and the balance between self-renewal and neurogenesis during cortical development. To identify the downstream signaling mechanisms involved.
Related to Chapter 1 of the results section.

OBJECTIVE 2. To investigate the role of CB_1 receptor signaling in corticofugal projection neuron development from a molecular, anatomical and functional perspective. This objective was approached *in vivo* by means of genetic manipulation of CB_1 receptors, and by exploring the potential impact of prenatal THC exposure in corticospinal motor neuron development.
Related to Chapter 2 of the results section.

OBJECTIVE 3. To characterize CB_1 signaling-mediated regulation of cortical pyramidal neuron radial migration and to dissect the molecular mechanisms implicated. To address the possible chemoattractant profile of endocannabinoids for radially migrating pyramidal neuroblasts.
Related to Chapter 3 of the results section.

3. RESULTS

a. **CHAPTER 1.** Signal transduction mechanisms responsible of endocannabinoid regulation of pyramidal precursor cell identity, proliferation and neurogenesis. Role of the PI3K/Akt/mTORC1 signaling cascade.

Original research article 1. Díaz-Alonso J, Aguado T, de Salas-Quiroga A, Ortega Z, Guzmán M, Galve-Roperh I. "CB_1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex". Cereb Cortex. 2014 Mar 7. [Epub ahead of print]

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CB_1 Cannabinoid Receptor-Dependent Activation of mTORC1/Pax6 Signaling Drives Tbr2 Expression and Basal Progenitor Expansion in the Developing Mouse Cortex

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The CB_1 cannabinoid receptor regulates cortical progenitor proliferation during embryonic development, but the molecular mechanism of this action remains unknown. Here, we report that CB_1-deficient mouse embryos show premature cell cycle exit, decreased Pax6- and Tbr2-positive cell number, and reduced mammalian target of rapamycin complex 1 (mTORC1) activation in the ventricular and subventricular cortical zones. Pharmacological stimulation of the CB_1 receptor in cortical slices and progenitor cell cultures activated the mTORC1 pathway and increased the number of Pax6- and Tbr2-expressing cells. Likewise, acute CB_1 knockdown in utero reduced mTORC1 activation and cannabinoid-induced Tbr2-positive cell generation. Luciferase reporter and chromatin immunoprecipitation assays revealed that the CB_1 receptor drives Tbr2 expression downstream of Pax6 induction in an mTORC1-dependent manner. Altogether, our results demonstrate that the CB_1 receptor tunes dorsal telencephalic progenitor proliferation by sustaining the transcriptional activity of the Pax6–Tbr2 axis via the mTORC1 pathway, and suggest that alterations of CB_1 receptor signaling, by producing the missexpression of progenitor identity determinants may contribute to neurodevelopmental alterations.

Keywords: basal progenitors, cannabinoid, corticogenesis, Eomes, mTORC1

Introduction

In the mammalian developing cortex, neural progenitor cells are differentially distributed in the ventricular and subventricular zones (VZ and SVZ). Apical and basal progenitor cells are identified by their selective expression of the transcription factors Pax6 and Tbr2/Eomes, respectively (Guillemot et al. 2006; Osumi et al. 2008). On one hand, the heterogeneity of apical VZ progenitor subpopulations contributes to the acquisition of the projection neuron diversity of the mature six-layered neocortex (Gal et al. 2006). In addition, intermediate/basal progenitors in the mouse SVZ can undergo additional rounds of division and contribute to the number and diversity of pyramidal neurons in the mature mammalian neocortex (Noctor et al. 2004; Hansen et al. 2010). Pax6, a paired domain-containing transcription factor, master regulates the network of neural stem cell decision-making genes (Sansom et al. 2009; Asami et al. 2011), being essential for the maintenance of the VZ radial glial cell pool and the transition from apical to basal progenitors by driving Tbr2 expression (Warren et al. 1999; Englund et al. 2005). Likewise, Tbr2 is essential for neuronal amplification and tightly controls the balance between neural stem cell renewal and neurogenesis (Guillemot et al. 2006). Thus, the Pax6–Tbr2 transcription factor axis exerts a pivotal role in the maintenance of cortical progenitor cell populations and in the appropriate timing of cortical neurogenesis (Englund et al. 2005; Sessa et al. 2008).

Whereas these and other endogenous fate determinants have been extensively studied (Guillemot et al. 2006), how extrinsic signals from neurogenic niches tune the appropriate coordination of neuronal generation, maturation and circuit establishment remains largely unknown. Synaptic and nonsynaptic neuronal activities constitute physiological inputs that tune neurogenesis during development and in the adult brain (Ge et al. 2007; Ben-Ari 2008). Thus, different neurotransmitters and neuromodulators have been shown to influence neural progenitor proliferation and neurogenesis. In particular, the CB_1 cannabinoid receptor, the most important mediator of endocannabinoid actions on key neurobiological processes (Castillo et al. 2012), exerts a regulatory role in neuronal differentiation and long-range connectivity (Berghuis et al. 2007; Mulder et al. 2008; Diaz-Alonso et al. 2012). In addition, CB_1 receptors are known to control neural progenitor cell proliferation in vitro and in vivo, both in the developing brain and in adult neurogenic areas (Jin et al. 2004; Aguado et al. 2005, 2007; Trazzi et al. 2010). However, the molecular mechanism of CB_1 receptor action on cortical progenitor expansion during embryonic development remains unknown.

Here, we show that CB_1 receptor signaling exerts a crucial role in mouse cortical progenitor expansion by promoting the generation of basal progenitors from radial glial cells. This CB_1 receptor-mediated effect occurs through the induction of Pax6 transcriptional activity via the mammalian target of rapamycin complex 1 (mTORC1) pathway. Thus, gain or loss of CB_1 receptor function enhanced or reduced, respectively, the activation of the mTORC1 pathway and $Pax6^+$ radial glial cell progression to Tbr2-expressing basal progenitors. These findings contribute to unveiling the molecular basis of cortical progenitor cell generation and may provide mechanistic clues to understand the origin of neurodevelopmental alterations originated by unbalanced progenitor expansion.

Materials and Methods

Materials

The following materials were kindly donated: Tbr2-promoter luciferase construct (M. Götz and L. Pinto, Ludwig Maximilians University Munich, Germany), Pax6 transcriptional activity reporter constructs (A. Stoykova and T. Tuoc, Max Plank Institute for Biophysical Chemistry, Göttingen, Germany), Pax6 expression vector (M. Nieto, National Center Biotechnology, Madrid, Spain), $CB_1^{-/-}$ and $CB_1^{f/f,\ Nex-Cre/+}$ colony-founding mice and CB_1 in situ hybridization (ISH) probes (B. Lutz and C. Hoffmann, Mainz, Germany), and HU-210 (R. Mechoulam, Hebrew University, Jerusalem, Israel).

Animals

Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with the European Commission regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Mice were maintained in standard conditions, keeping littermates grouped in breeding cages, at a constant temperature (20 ± 2 °C) on a 12-h light/dark cycle with food and water ad libitum. The generation and genotyping of $CB_1^{-/-}$ and $CB_1^{f/f,\ Nex-Cre/+}$ (Nex-$CB_1^{-/-}$), and their respective wild-type (WT) littermate controls, has been reported elsewhere and was performed accordingly (Monory et al. 2006). Mouse embryonic tissues were obtained upon timed mating as assessed by vaginal plug observation (E0.5). For CB_1 expression analyses, we compared WT, $CB_1^{-/-}$, $CB_1^{f/f}$, and Nex-$CB_1^{-/-}$ embryos. After carefully ascertaining that WT and $CB_1^{f/f}$ expression levels were indistinguishable, only the comparison among $CB_1^{f/f}$, Nex-$CB_1^{-/-}$, and $CB_1^{-/-}$ embryos is shown in Figure 1.

Immunofluorescence and Confocal Microscopy

Cell proliferation was determined after intraperitoneal iodo- and bromo-deoxyuridine (IdU, BrdU) injection (100 µg/g body weight) of pregnant females at E12.5 and E13.5 as indicated. Coronal brain slices (14-µm thick) were processed as previously described (Diaz-Alonso et al. 2012). Cortical layers were identified by their discrete cell densities as visualized by DAPI counterstaining.

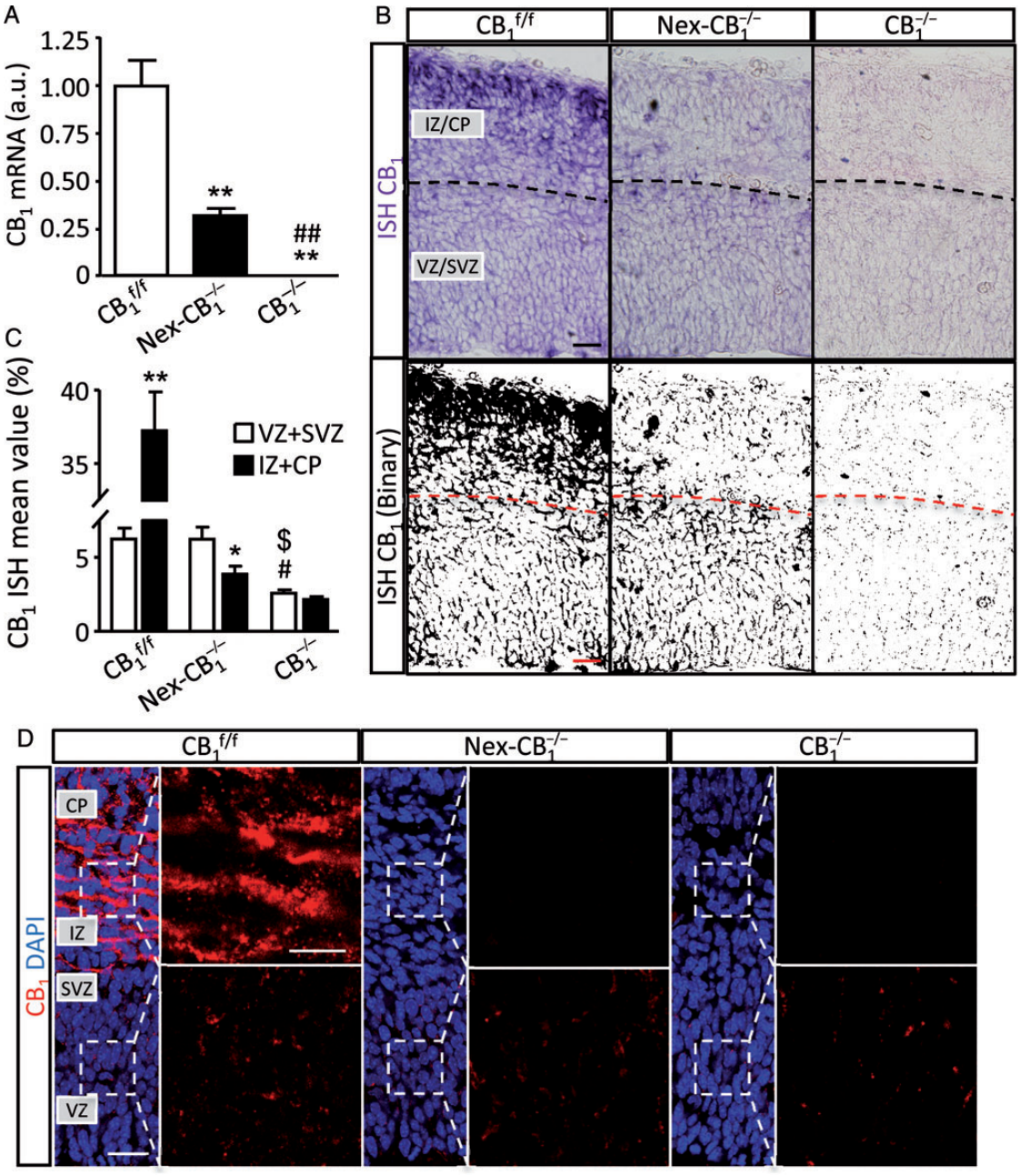

Figure 1. The CB_1 receptor is expressed, albeit at low levels, in proliferative areas of the developing mouse cortex. (*A*) CB_1 mRNA was quantified by qPCR in E13.5 cortical extracts of $CB_1^{f/f}$, Nex-$CB_1^{-/-}$, and $CB_1^{-/-}$ embryos ($n = 4$ for each group). $^{**}P < 0.01$ versus $CB_1^{f/f}$ extracts; $^{\#\#}P < 0.01$ versus Nex-$CB_1^{-/-}$ extracts. (*B* and *C*) Representative raw and binary ISH images in the developing cortex of the same 3 genotypes at E14.5. Semiquantitative analysis of CB_1-transcript levels was performed to estimate the relative presence of CB_1 transcripts in each compartment ($n = 4$ for each group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the respective VZ+SVZ; $^{\#}P < 0.05$ versus Nex-$CB_1^{-/-}$ VZ+SVZ; $^{\$}P < 0.05$ versus $CB_1^{f/f}$ VZ +SVZ (*D*) Representative images of CB_1 receptor immunoreactivity in E14.5 $CB_1^{f/f}$, Nex-$CB_1^{-/-}$, and $CB_1^{-/-}$ cortical sections. Cell nuclei were counterstained with DAPI. Insets of the indicated areas are shown. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone. Scale bars: *B*, 25; *D*, 25 and 10 µm (insets).

Immunofluorescence was performed, after blockade with 5% goat serum, by overnight incubation at 4 °C with the indicated primary antibodies (Table 1), followed by incubation for 1 h at room temperature with secondary antibodies. The appropriate anti-mouse, rat, guinea pig, and rabbit highly cross-adsorbed AlexaFluor 488, AlexaFluor 546, Alexa Fluor 594, and AlexaFluor 647 secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with 2 passes by Kalman filter and a 1024 × 1024 collection box. Immunofluorescence data were obtained in a double blind manner by an independent observer, and all quantifications were obtained from a minimum of 6 sections from 1-in-10 series per mice. Immunofluorescence of cortical sections was performed along the rostral to caudal axis and the quantifications were carried out in equivalent sections from the mediolateral area of the rostro-medial cortex. Quantifications of cortical progenitor cell populations in the VZ/SVZ were performed in a 275-μm-wide 180-μm-high cortical box. Positive cells for the corresponding markers were quantified. In CB_1 receptor knockdown experiments by in utero and ex vivo electroporation, cells positive for the indicated markers were quantified within the GFP^+ cell population.

In Situ Hybridization

Coronal sections (14 μm) of E14.5 or E16.5 mouse embryonic brains were obtained and processed for ISH as described (Diaz-Alonso et al. 2012). Representative ISH images were processed with Image J software, and binary images were obtained after application of the same background subtraction. Binary images were employed for semiquantitative determination of the ISH signal present in the different cortical compartments. In some cases, CB_1 ISH was followed by immunofluorescence detection of the GFP protein (as described above) to identify the electroporated area.

In Utero and Ex Vivo siRNA Electroporation

$siCB_1$ and siControl (Thermo Scientific) were electroporated together with pCAG-GFP and Fast Green in the lateral ventricle of E13.5 embryos, either ex vivo, followed by coronal slicing and organotypic culture for 2 days in vitro (DIV), or in utero, as described (Diaz-Alonso et al. 2012). In utero electroporated embryos were analyzed 3 days later, at E16.5.

Primary Cortical Progenitor and Organotypic Cultures

Cortical progenitors were obtained from dissected cortices isolated from E13.5 $CB_1^{f/f}$ embryos and grown as neurospheres as previously described (Aguado et al. 2005). For pharmacological regulation experiments, neurospheres were dissociated with accutase (Sigma) and mechanically, plated on polylysine and laminin-coated coverslips at a density of 50 000 cells/cm^2, and grown in a chemically defined medium composed of DMEM/Hams F-12 medium supplemented with N2 (Millipore), glutamine and basic FGF. For acute CB_1 receptor genetic ablation studies, $CB_1^{f/f}$ neurospheres were subjected to nucleofection (Amaxa nucleofector, Lonza, Spain) with either pCAG-Cre-GFP or pCAG-GFP prior to plating. Proliferation assays were performed after 1 day of preincubation with BrdU (10 μg/mL). The progenitor identity was assessed after 2 DIV by quantification of highly immunoreactive $Tbr2^+$ cells from ≥10 randomly selected view fields/coverslip after DAPI counterstaining. Slice cultures were maintained in semidry conditions on Millicell membranes (Millipore) containing neurobasal medium, B27 (Invitrogen, 1%), N2 (1%), glutamine (1%), and penicillin/streptomycin (1%). Pharmacological manipulation was performed at 1 DIV with the indicated drugs at the indicated concentrations. CB_1 receptor expression rescue in $CB_1^{-/-}$ neural progenitors was performed by nucleofection with pCAG-CB_1-GFP or pCAG-GFP as a control.

Table 1
Antibodies employed in this study

Antigen	Species	Source	Dilution
BrdU	Rat monoclonal	Abcam	1:250
BrdU/IdU	Mouse monoclonal	Becton/Dickinson	1:200
CB_1	Rabbit polyclonal	Frontier Institute	1:500
CB_1	Guinea pig polyclonal	Frontier Institute	1:500
GFP	Rabbit polyclonal	Abcam	1:500
Ki-67	Rabbit polyclonal	Neomarkers	1:250
Nestin	Mouse monoclonal	Chemicon	1:200
Pax6	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:50
Pax6	Rabbit polyclonal	Millipore	(10 μg ChIP reaction)
PCNA	Mouse monoclonal	Abcam	1:1000
RC2	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:50
pS6	Rabbit polyclonal	Cell Signaling	1:250 (IF) 1:1000 (WB)
pS6-AlexaFluor488 conjugate	Rabbit polyclonal	Cell Signaling	1:100
Tbr2	Rabbit polyclonal	Abcam	1:500
α-Tubulin	Mouse monoclonal	Sigma	1:4000 (WB)

Gene Promoter Activity Assays

Primary cortical progenitors were transiently transfected with the Tbr2-promoter luciferase reporter constructs (Pinto et al. 2009) by nucleofection, and P19 mouse embryonic carcinoma cells were transiently transfected (Lipofectamine 2000, Invitrogen) with a Pax6 expression vector and a Pax6-binding sequences (pCON/P3) reporter construct (Tuoc and Stoykova 2008). Firefly and renilla (as internal transfection control) luciferase activities were measured using Dual-Luciferase Reporter assay system (Promega) in a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Real-Time Quantitative PCR and Chromatin Immunoprecipitation

RNA was isolated using RNeasy Plus kit (Quiagen). cDNA was obtained with Transcriptor (Roche). Real-time quantitative PCR (qPCR) assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA and β-actin levels as reference.

For chromatin immunoprecipitation (ChIP) analysis, WT and $CB_1^{-/-}$ cortices were dissected from E14.5 mouse embryos and diced in ice-cold Hanks' buffered saline solution. Samples were processed as recommended by the manufacturer (EZ-ChIP, Millipore) and immunoprecipitation was performed with an anti-Pax6 antibody (Millipore) or a nonspecific rabbit IgG as control. Pax6-bound DNA was determined by qPCR analysis. Primers were designed from published data for the amplification of Pax6-binding DNA sequences (Sansom et al. 2009) in the promoter region of several genes (Tbr2, NF1, neurogenin3, Hes7, and Rap1b). Reactions were performed in triplicate on 3 independent ChIP samples per genotype ($n = 12$ cortices for each genotype). The enrichment for each gene was calculated by normalizing the Pax6/input ratio with the Pax6/input ratio of a DNA region that does not bind Pax6 (Gad1 gene promoter).

Data Analyses and Statistics

Results shown represent the means ± SEM, and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way ANOVA, as appropriate. A post hoc analysis was made by the Student–Neuman–Keuls test.

Results

The CB_1 Cannabinoid Receptor is Expressed, Albeit at Low Levels, in Proliferative Areas of the Developing Mouse Cortex

To investigate the role of CB_1 receptor signaling in cortical progenitor cell populations, it was crucial to determine first the

expression levels of this receptor in the VZ/SVZ. Previous studies had indicated the presence of CB_1 receptors in proliferative cells of the mouse developing cortex in vivo and in vitro (Aguado et al. 2005; Mulder et al. 2008). However, the expression levels of the CB_1 receptor in the cortical VZ/SVZ in vivo may have been overestimated owing to the recently described lack of specificity of commercially available anti-CB_1 antibodies (Morozov et al. 2013). We quantified CB_1 transcripts by qPCR in cortical extracts derived from $CB_1^{f/f}$, conditional Nex-$CB_1^{-/-}$, and complete $CB_1^{-/-}$ embryos at E13.5. CB_1 transcripts were undetectable in $CB_1^{-/-}$ samples, while low but significant CB_1-transcript levels were consistently observed in Nex-$CB_1^{-/-}$ cortical extracts (Fig. 1*A*). This remainder (~25%) of CB_1 transcripts present in the latter samples conceivably corresponds to progenitor cells owing to the postmitotic neuron selectivity of Nex-driven recombinase expression (Goebbels et al. 2006; Diaz-Alonso et al. 2012).

The presence of CB_1 transcripts in the developing mouse cortex was also analyzed by ISH (Fig. 1*B*). CB_1 receptor expression in $CB_1^{f/f}$ animals showed a typical inside-out expression gradient, with higher levels in postmitotic cells. In addition, CB_1 transcripts were present, albeit at low levels, in the VZ/SVZ. The CB_1-transcript signal was essentially absent in complete $CB_1^{-/-}$ mice, thus confirming the specificity of the CB_1 ISH. Semiquantitative analysis of the ISH signal was subsequently performed to estimate the relative presence of CB_1 transcripts in the progenitors' compartments (VZ/SVZ) and the postmitotic areas (IZ/CP) (Fig. 1*C*). Using astringent image acquisition and processing settings, the CB_1 ISH signal in $CB_1^{f/f}$ within the VZ/SVZ accounted for a ~17% of total (VZ/SVZ + IZ/CP) CB_1 signal, a value that is not far from that of CB_1 mRNA levels remaining in the Nex-$CB_1^{-/-}$ cortices as determined by qPCR (Fig. 1*A*). Of note, in Nex-$CB_1^{-/-}$ mice, the VZ/SVZ compartment preserved its CB_1 mRNA expression (Fig. 1*C*, VZ+SVZ white columns) and only postmitotic neurons (Fig. 1*C*, IZ+CP black columns) lost receptor expression.

Similar to the aforementioned ISH analyses, the characterization of CB_1 receptor protein expression by confocal microscopy proved the efficient ablation of CB_1 in the postmitotic area of Nex-$CB_1^{-/-}$ and $CB_1^{-/-}$ mouse cortices (Fig. 1*D*, upper panels). In line with the ISH data, in the VZ/SVZ of $CB_1^{f/f}$ and Nex-$CB_1^{-/-}$ animals, the presence of CB_1 receptors was low but clearly distinguishable from the faint unspecific reactivity observed with the anti-CB_1 antibody in $CB_1^{-/-}$ mice (Fig. 1*D*, lower panels) (Morozov et al. 2013).

The CB_1 Cannabinoid Receptor Regulates Cell Cycle Maintenance and Cortical Progenitor Cell Populations

To determine the consequences of the loss of CB_1 receptor signaling on the expansion of cortical progenitors in vivo, we analyzed cell cycle progression by double-labeling of cycling progenitors with IdU and BrdU injections at E12.5 and E13.5, respectively (Fig. 2*A*). In CB_1-deficient cortices, there was a decreased fraction of double-positive IdU^+BrdU^+ cycling progenitors within the IdU^+ population at E14.5. Likewise, cell cycle maintenance of cortical progenitors between E13.5 and E14.5, identified as the fraction of $BrdU^+$ cells that were immunoreactive for Ki67, an endogenous marker of proliferating cells, was also reduced in the SVZ of $CB_1^{-/-}$ embryos (Fig. 2*B*,*C*). These results are in agreement with the proposed role of CB_1 receptor signaling in neural progenitor cell proliferation (Aguado et al. 2005; Mulder et al. 2008) and indicate the involvement of the receptor in the regulation of cell cycle maintenance of cortical progenitor cell populations.

To evaluate the involvement of CB_1 receptor signaling in cortical progenitor expansion in vivo, we quantified the $Pax6^+$ and $Tbr2^+$ progenitor cell populations in CB_1 receptor knockout mice and WT littermates. $Pax6^+$ apical progenitor cell number was reduced in the VZ/SVZ of $CB_1^{-/-}$ mice at E14.5 (Fig. 2*D*,*E*), which was accompanied by a reduced $Tbr2^+$ intermediate progenitor pool (Fig. 2*F*,*G*). Remarkably, at E16.5, decreased $Pax6^+$ and $Tbr2^+$ cell populations were still evident in $CB_1^{-/-}$ embryos. Pax6 and Tbr2 mRNA levels were also reduced in the absence of CB_1 receptors (Pax6: 1.00 ± 0.09 and 0.61 ± 0.04, $P \leq 0.01$; Tbr2: 1.00 ± 0.14 and 0.55 ± 0.11, $P \leq 0.05$; WT and $CB_1^{-/-}$, respectively). The reduction of cortical progenitor cell populations in CB_1-deficient embryos was also reflected in a decreased immunoreactivity of the RC2 and nestin markers (Fig. 2*H*,*J*). In contrast, CB_1 ablation in postmitotic neurons in the Nex-$CB_1^{-/-}$ mice does not affected the proliferation of neural progenitors (Diaz-Alonso et al. 2012), the $Pax6^+$ and the $Tbr2^+$ cell populations (Supplementary Fig. 1). In summary, these findings suggest that CB_1 receptor signaling regulates cortical progenitor expansion in the VZ/SVZ of the developing telencephalon. This effect involves, at least in part, a cell-autonomous role of CB_1 receptors in cortical progenitors, although it is also conceivable the additional contribution by an indirect mechanism of CB_1 receptors present in postmitotic neurons.

The CB_1 Cannabinoid Receptor Promotes Pax6–Tbr2 Cortical Progenitor Expansion

To investigate the role of CB_1 receptor signaling in cortical progenitors, we performed pharmacological manipulation studies in organotypic cultures of E13.5-WT embryonic brain cortices. The synthetic cannabinoid agonist HU-210 increased apical ($Pax6^+$) and basal ($Tbr2^+$) progenitor cell populations in a CB_1 receptor-dependent manner, as evidenced by the ability of the CB_1 antagonist rimonabant (SR141716) to prevent cannabinoid-induced increase in progenitor cell number and mRNA levels (Fig. 3*A*,*D*). We confirmed the involvement of CB_1 receptor signaling in the regulation of the Pax6–Tbr2 transcription factor axis by siRNA interference assays. Thus, CB_1 receptor knockdown by $siCB_1$ ex vivo electroporation (Supplementary Fig. 2) was accompanied by reduced Pax6 and Tbr2 mRNA levels (Fig. 3*E*). The transcript levels of Sox2, a transcription factor essential for neural stem cell proliferation and self-renewal (Guillemot et al. 2006), were also reduced in $siCB_1$-electroporated cortices. Moreover, HU-210-induced increase in $Tbr2^+$ cell number in cortical slices was prevented by $siCB_1$ electroporation (Fig. 3*F*,*G*). To further support the role of CB_1-mediated signaling in cortical progenitor expansion in vivo, acute elimination of CB_1 receptors was conducted by in utero electroporation of $siCB_1$ or siControl at E13.5, and embryos were analyzed at E16.5. Importantly, ablation of CB_1 receptor signaling reduced the $Tbr2^+$ population within the GFP^+ cell population when compared with siControl-electroporated embryos (Fig. 3*H*,*I*).

The CB_1 Cannabinoid Receptor Regulates mTORC1 Signaling in Progenitors of the Developing Mouse Cortex In Vivo

To study the mechanism of CB_1-mediated regulation of cortical progenitors in vivo, among the various signaling pathways activated by CB_1 receptors (Harkany et al. 2007), we analyzed

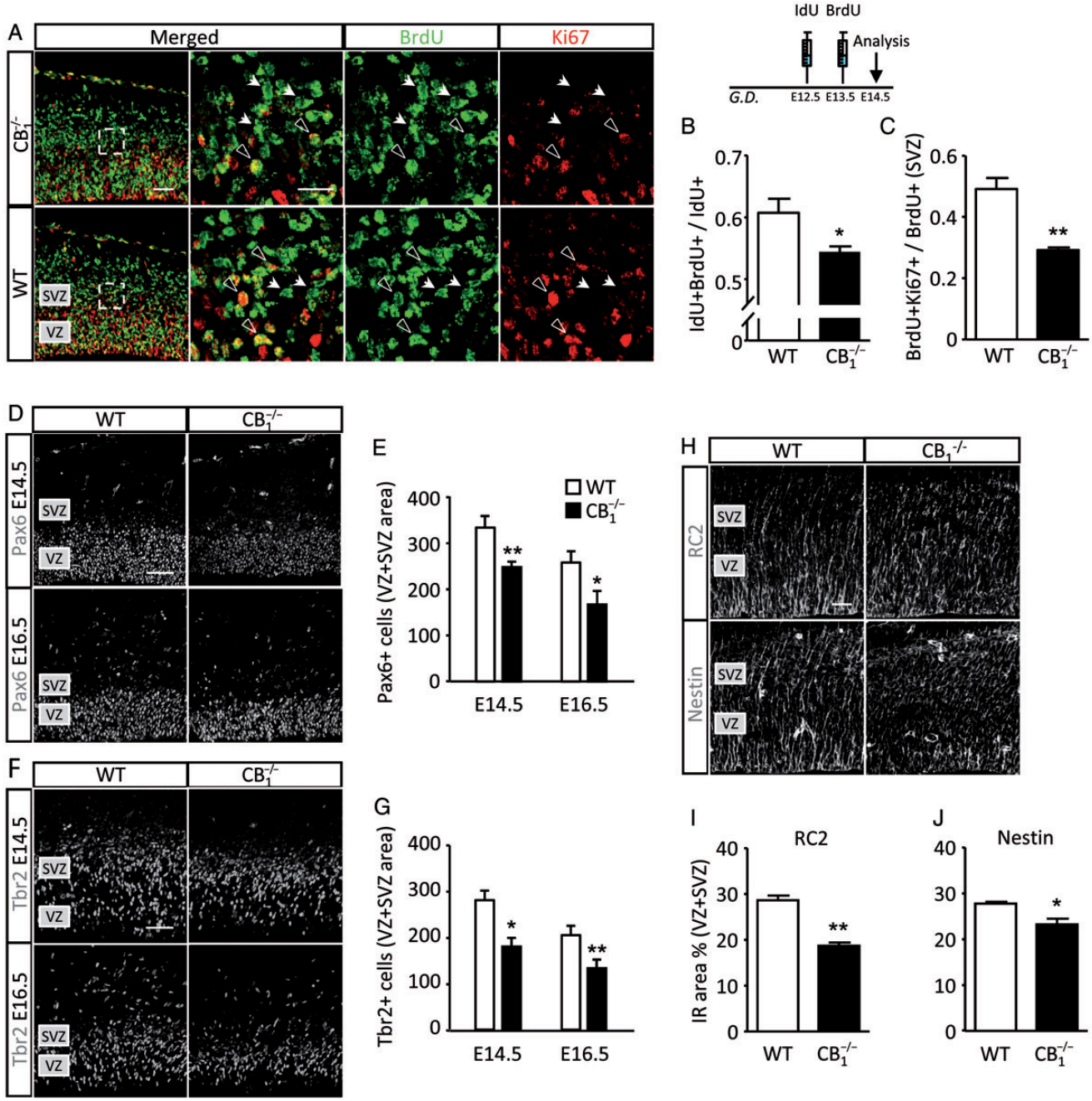

Figure 2. The CB_1 receptor controls neural progenitor populations and cell cycle during cortical development. (*A–C*) Pregnant $CB_1^{+/-}$ female mice crossed with $CB_1^{+/-}$ male mice were injected with IdU and BrdU at E12.5 and E13.5 gestational days (GD), respectively, and embryonic cortices analyzed at E14.5. Quantification of IdU^+BrdU^+/IdU^+ and $BrdU^+Ki67^+/BrdU^+$ cells was performed, allowing to assess cell cycle maintenance ratio between E12.5 and E13.5 and between E13.5 and E14.5, respectively ($n = 4$ for each group). Representative images of $BrdU^+$ (green) and $Ki67^+$ (red) cell distribution at E14.5 are shown. Empty arrowheads indicate double-positive cells, white arrows point BrdU-only cells. (*D–G*) Quantification of highly immunoreactive Pax6- and Tbr2-expressing cells in a 275-μm-wide 180-μm-thick box placed adjacent to the ventricular wall (covering the VZ and SVZ) of $CB_1^{-/-}$ and WT littermates at E14.5 and E16.5 ($n = 7$ and 8; 6 and 5, respectively, for each group). Representative images are shown. (*H–J*) Quantification of nestin- and RC2-immunoreactive (IR) area in the VZ/SVZ of $CB_1^{-/-}$ and WT littermates at E14.5 ($n = 3$ for each genotype). Representative images are shown. Scale bars: *C*, 50 and 20 μm (insets); *D* and *F*, 50 μm; *H*, 25 μm. $^*P < 0.05$; $^{**}P < 0.01$ versus WT mice.

the mTORC1 pathway, as the CB_1 receptor is known to activate mTORC1 in neurons (Puighermanal et al. 2009), and this pathway exerts key pleiotropic actions in the control of neural cell fate during brain development (Crino 2011). The mTORC1 pathway was found to be active in the VZ/SVZ of the developing cortex, as evidenced by the phosphorylation of the ribosomal protein S6 at Ser 235/236, a canonical readout of mTORC1 activation, and genetic inactivation of CB_1 receptor signaling reduced the number of phospho-$S6^+$ cells in the VZ and SVZ of E14.5 cortices (Fig. 4*A*,*B*). Proliferating cells in the VZ/SVZ co-localized to a large extent with S6 phosphorylation, and loss of CB_1 receptor function decreased phospho-S6 immunoreactive $Ki67^+$ cycling progenitors of the VZ/SVZ (Fig. 4*C*,*D*). Double immunofluorescence with an anti-Pax6 or anti-Tbr2 antibody,

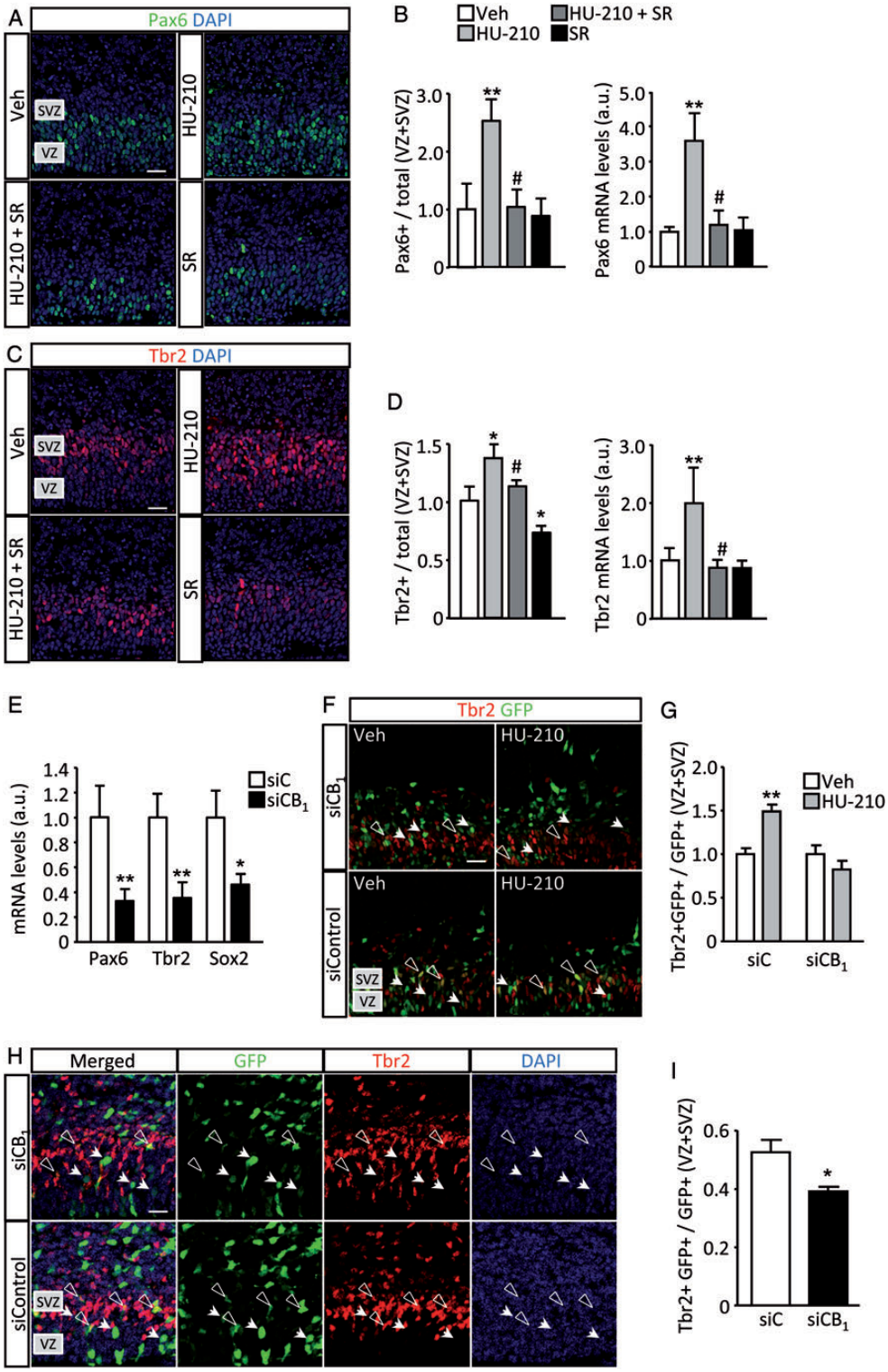

Figure 3. CB_1 receptor signaling drives $Pax6^+$ and $Tbr2^+$ cortical progenitor expansion in organotypic cultures. (*A–D*) Cortical organotypic cultures from E14.5-WT cortices were exposed for 48 h to vehicle (Veh) or HU-210 (1 μM), alone or combined with SR141716 (SR, 25 μM), and highly immunoreactive Pax6- and Tbr2-positive cells (green and red, respectively) quantified in the ventricular and subventricular zones (VZ and SVZ). Representative immunofluorescence images are shown. Pax6 and Tbr2 mRNA levels were also determined by qPCR after pharmacological manipulation of cortical slices (*B* and *D*, right panels). (*E*) Organotypic cultures prepared from CB_1 siRNA ($siCB_1$) or control siRNA (siC) and pCAG-GFP-electroporated E14.5-WT cortices and the transfected (GFP^+) areas were dissected and analyzed after 48 h by qPCR for the indicated transcripts. (*F–G*) WT cortices were electroporated ex vivo at E14.5 with $siCB_1$ or siControl and pCAG-GFP, after 1 day in vitro (DIV), treated with HU-210 (1 μM) or vehicle. $Tbr2^+$ cells (red) were quantified in the transfected GFP^+ cell population after 2 DIV. Empty arrowheads indicate double-positive cells, white arrows point GFP-only cells. Results are represented as the $Tbr2^+GFP^+$ cells/total GFP^+ cells ratio in HU-210-treated slices normalized to the corresponding vehicle-treated slices. Results correspond to 4 independent experiments. (*H–I*) In utero electroporation of siControl or $siCB_1$ together with pCAG-GFP was performed at E13.5 and $Tbr2^+$ cells (red) in the ventricular and subventricular zone (VZ/SVZ) were quantified at E16.5 and referred to the GFP^+ electroporated cell population. Empty arrowheads indicate double-positive cells, white arrows point GFP-only cells. Embryos ($n = 3$) from at least 2 different litters per condition. $^*P < 0.05$; $^{**}P < 0.01$ versus vehicle-treated or control siRNA-electroporated slices. a.u., arbitrary units. Scale bars: 25 μm.

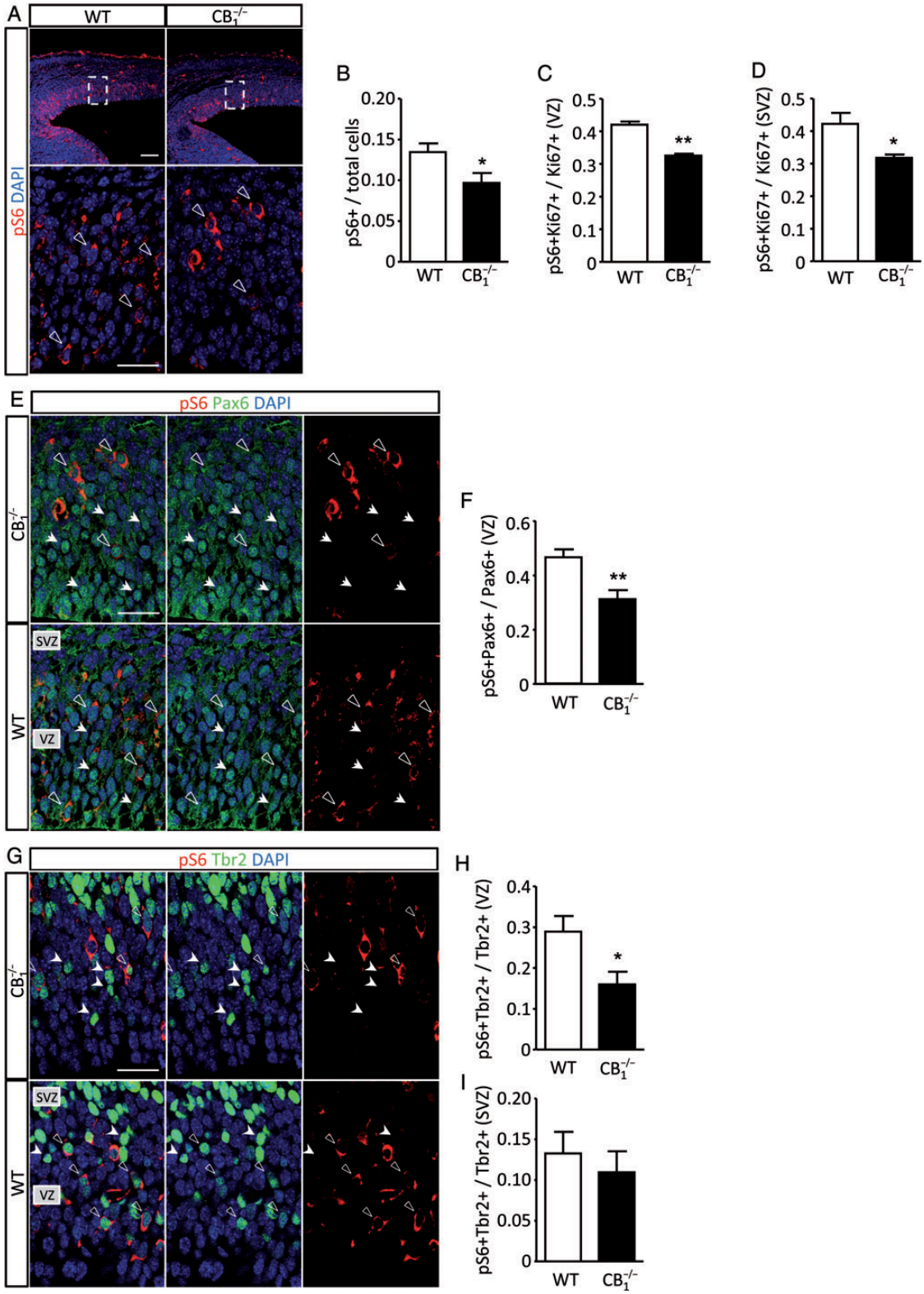

Figure 4. The CB_1 receptor controls mTORC1 signaling in cortical progenitors in vivo. (*A* and *B*) Phospho-S6-positive (pS6, red) cells were quantified after immunofluorescence in the developing dorsal telencephalon of $CB_1^{-/-}$ and WT littermates at E14.5 and referred to total cell number in the analyzed cortical column. Empty arrowheads point to representative pS6+ cells. (*C* and *D*) mTORC1 activation status in proliferative progenitor cells was assessed by double immunofluorescence analysis of pS6 and Ki67 in the VZ and SVZ of E14.5 $CB_1^{-/-}$ and WT littermates ($n = 3$ embryos for each genotype). (*E* and *F*) mTORC1 signaling in $Pax6^+$ radial glial cells was assessed by the quantification of phospho-$S6^+$ cells highly immunoreactive for Pax6 (green) and referred to Pax6 highly immunoreactive total cell number in the VZ. Empty arrowheads point to $Pax6^+pS6^+$ cells, whereas white arrows indicate $Pax6^+pS6^-$ cells ($n = 10$ embryos for each group). (*G–I*) mTORC1 activation status in intermediate progenitor cells labeled with an anti-Tbr2 antibody (green) was assessed with pS6 immunoreactivity in the VZ and SVZ (*H* and *I*, respectively) of $CB_1^{-/-}$ and WT littermates ($n = 6$ for each group). Empty arrowheads point to $Tbr2^+pS6^+$ cells, whereas white arrows indicate $Tbr2^+pS6^-$ cells. $^*P < 0.05$; $^{**}P < 0.01$ versus WT mice. Scale bars: *A* 100 and 25 μm (insets); *E* and *G*, 25 μm.

together with an anti-phospho-S6 antibody, showed that a large fraction of Pax6$^+$ cells have an active mTORC1 pathway (47 ± 3% Pax6$^+$pS6$^+$/Pax6$^+$; $n = 10$), while mTORC1 activity in Tbr2$^+$ cells of the SVZ cells was notably lower (13 ± 4% Tbr2$^+$pS6$^+$/Tbr2$^+$; $n = 6$). Interestingly, ablation of the CB_1 receptor decreased the activation of the mTORC1 pathway in VZ Pax6$^+$ cells (Fig. 4*E*,*F*) as well as in the recently generated Tbr2$^+$ cells located at the VZ (Fig. 4*G*,*H*), but not in the SVZ (Fig. 4*I*). These results indicate that CB_1 receptor-driven mTORC1 activity contributes to the apical to basal progenitor transition.

The CB_1 Cannabinoid Receptor Activates mTORC1 Signaling in Proliferating Cortical Progenitors

To investigate the mechanism of CB_1 receptor-induced expansion of neural progenitor cells, we performed pharmacological manipulation experiments in E13.5 progenitor cultures. First, we confirmed that CB_1 receptors regulate cortical progenitor identity in vitro in a similar manner than in vivo. Treatment with HU-210 increased the generation of Tbr2$^+$ cells, and SR141716 prevented this effect (Fig. 5*A*,*B*). As revealed by immunofluorescence (Fig. 5*C*,*D*), HU-210 increased S6 phosphorylation in proliferating cells that express the endogenous marker PCNA. HU-210-induced S6 phosphorylation in cortical progenitor cultures was prevented by co-administration of SR141716 or rapamycin (a widely employed mTORC1 inhibitor). Western blot analysis confirmed the activation of the mTORC1 pathway by CB_1 signaling in cortical progenitor cells. Thus, the HU-210-induced increase of phosphorylated S6 was prevented by SR141716 and rapamycin (Fig. 5*E*,*F*). In addition, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294,002 and the MEK inhibitor UO126 prevented HU-210-induced mTORC1 activation. To unequivocally address the role of CB_1 receptor signaling in the control of the mTORC1 pathway in neural progenitor cells, we performed an acute genetic ablation strategy by means of pCAG-Cre-GFP or pCAG-GFP nucleofection in $CB_1^{f/f}$-derived neurospheres. Loss of CB_1 receptor function reduced cell proliferation as quantified by BrdU$^+$ cell immunofluorescence within the GFP$^+$ cell population 2 days after nucleofection (Fig. 5*G*,*H*), and, in agreement with pharmacological gain of function experiments, CB_1 ablation by Cre-expressing vector nucleofection reduced phospho-S6$^+$ cell number (Fig. 5*I*,*J*).

We furthermore confirmed the involvement of CB_1 receptors in regulating mTORC1 activity in proliferating cells by ex vivo siRNA electroporation. CB_1 receptor knockdown prevented the HU-210-mediated increase in pS6$^+$ PCNA$^+$ cell number in cortical slices (Fig. 6*A*,*B*). As expected, inhibition of mTORC1 by rapamycin abrogated basal and HU-210-induced phospho-S6 cells (Supplementary Fig. 3). In addition, acute CB_1 knockdown in utero decreased pS6-positive cells within the transfected cell pool in the VZ, confirming the involvement of CB_1 receptors in the regulation of mTORC1 activity in cortical progenitor cells in vivo (Fig. 6*C*,*D*). Finally, mTORC1 inhibition by rapamycin blunted the HU-210-induced increase in Tbr2$^+$ cell number (Fig. 6*E*,*F*), confirming the importance of this signaling pathway in CB_1-induced basal progenitor expansion. In summary, these results demonstrate that CB_1 receptor signaling drives the expansion of cortical Tbr2$^+$ progenitor cells through the activation of the mTORC1 pathway, at least in part, in a cell-autonomous manner.

The CB_1 Cannabinoid Receptor Drives Tbr2 Expression Through the Stimulation of Pax6 Transcriptional Activity

To examine in detail the molecular mechanism of CB_1 receptor action in cortical progenitors, we sought to investigate its impact on the intrinsic determinants of neural progenitor identity. For this purpose, primary progenitor cell cultures were nucleofected with a luciferase construct under the control of the Tbr2 promoter (Pinto et al. 2009). CB_1 receptor activation by HU-210 increased Tbr2-promoter activity in WT, but not in CB_1--deficient cells (Fig. 7*A*). Importantly, rescue of CB_1 receptor expression in $CB_1^{-/-}$ progenitors after pCAG-CB_1 nucleofection restored basal and cannabinoid-induced Tbr2-promoter-driven luciferase activity (Fig. 7*B*). HU-210-induced Tbr2-promoter luciferase activity was prevented by SR141716 and rapamycin (Fig. 7*C*), pointing to the involvement of the mTORC1 pathway in CB_1 receptor-induced Tbr2 expression.

Considering the prominent role of the transcription factor Pax6 in cortical neurogenesis, its pivotal action in the transition from apical to basal progenitor cell populations and its role in driving Tbr2 expression (Englund et al. 2005; Tuoc and Stoykova 2008; Sansom et al. 2009; Georgala et al. 2011), we sought to investigate whether Pax6 is involved in CB_1 receptor-mediated upregulation of Tbr2 expression. Transfection of a construct with the Pax6 consensus DNA-binding sites of its paired-box and paired-like homeodomains (pCON and P3 sites, respectively) controlling luciferase expression was performed in P19 mouse embryonic carcinoma cells. HU-210 promoted Pax6 transcriptional activity in a CB_1 receptor-dependent manner, as shown by the prevention of HU-210-induced pCON/P3 activity by SR141716 co-incubation (Fig. 7*D*). In addition, rapamycin blunted HU-210-induced Pax6 activity, thus indicating that the CB_1 receptor drives Pax6 transcriptional activity in an mTORC1-dependent manner.

Next, we confirmed that Pax6 is an effective regulator of Tbr2 in our model. Pax6 overexpression in neural progenitors increased Tbr2-promoter luciferase activity (Fig. 7*E*). We subsequently analyzed Pax6 binding to the Tbr2 promoter by performing chromatin immunoprecipitation (ChIP) analysis in WT and CB_1-deficient embryonic cortical extracts. Pax6-bound DNA was obtained and qPCR analysis revealed a significant enrichment of the Tbr2 promoter in WT extracts (Fig. 7*F*) while, in CB_1-deficient extracts, Pax6 binding to the Tbr2 promoter was significantly reduced. Overall, these results support that CB_1 receptor signaling drives Tbr2 expression by increasing Pax6 transcriptional activity in an mTORC1-dependent manner.

Discussion

Here, we show that CB_1 receptor signaling in vivo and in vitro drives the expansion of cortical progenitor cells by inducing the activity of the Pax6–Tbr2 transcription factor cassette via mTORC1 signaling. Pharmacological and acute genetic CB_1 receptor manipulation evidenced that CB_1-mediated Tbr2 expression occurs downstream of Pax6 induction in an mTORC1-dependent manner. Thus, in the absence of CB_1 receptor signaling, the Tbr2$^+$ basal progenitor cell population is reduced as a consequence of decreased Pax6 activity. These findings delineate the signaling mechanism involved in CB_1 receptor-mediated regulation of cortical progenitor expansion in the developing telencephalon.

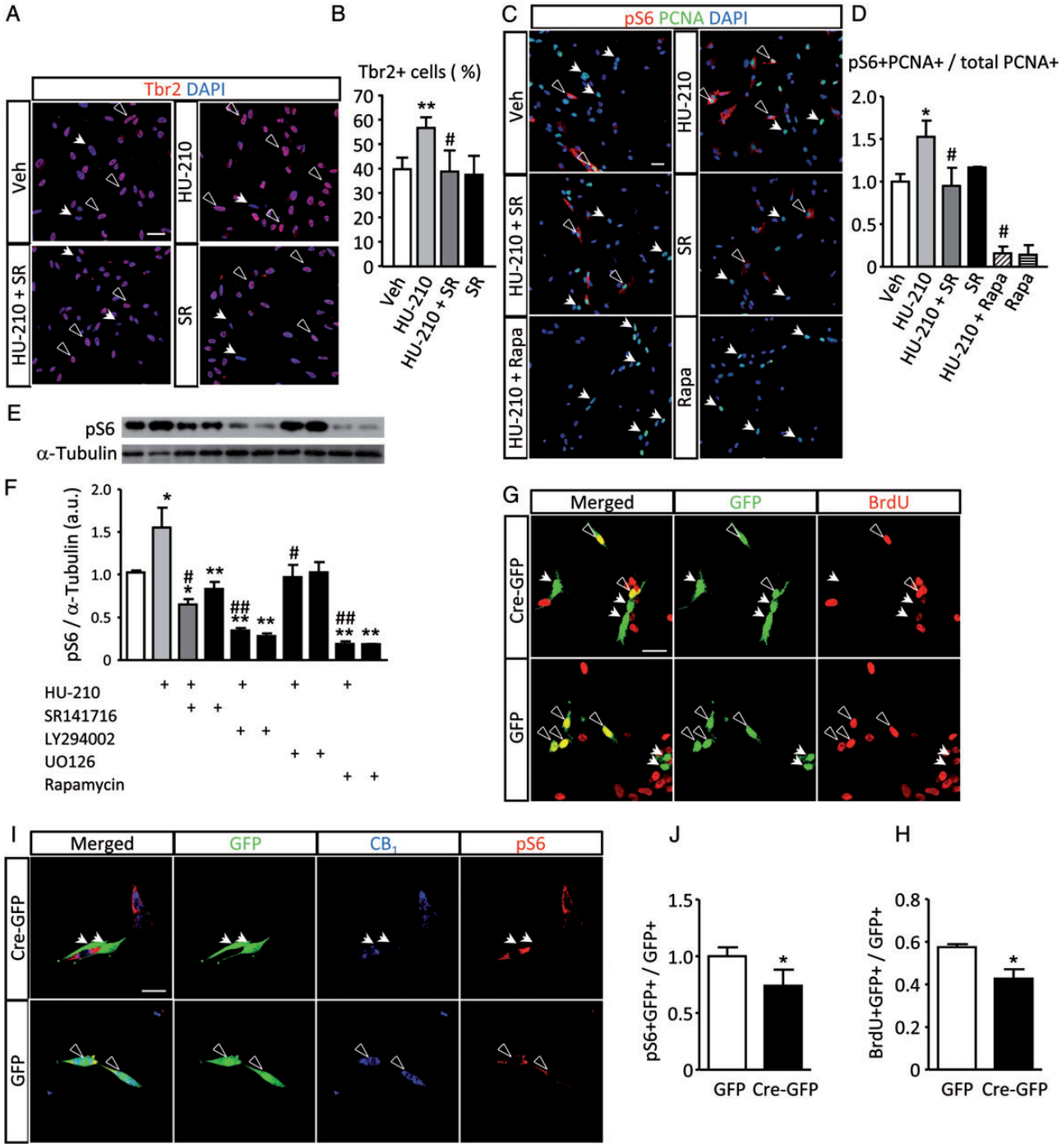

Figure 5. CB_1 receptor signaling regulates mTORC1 activity in proliferating cortical progenitors. (*A* and *B*) Cortical progenitor cultures from E14.5-WT embryos were treated for 24 h with vehicle (Veh) or HU-210 (100 nM), alone or combined with SR141716 (SR, 1 μM), and highly immunoreactive $Tbr2^+$ cells were quantified. Total cell numbers were assessed by DAPI counterstaining. Positive cells are indicated by empty arrowheads and Tbr2-negative cells by white arrows. (*C* and *D*) Cortical progenitors were treated as above with HU-210, alone or in the presence of SR or rapamycin (Rapa, 100 nM). Immunofluorescence was performed with antibodies against PCNA (green) and S6 phosphorylated at Ser235/236 (red) and $pS6^+$ cells were quantified in the $PCNA^+$ cell population. Representative double-positive cells are pointed by empty arrowheads, while PCNA-only positive cells are indicated by white arrows. (*E* and *F*) Western blot analysis was performed with anti-phospho-S6 antibody in cortical progenitor extracts after 30 min of CB_1 stimulation with HU-210 preceded, where indicated, by 1 h preincubation with CB_1 antagonist (SR), or PI3K (LY294-002), MEK (UO126), or mTORC1 (Rapa) inhibitors. Loading control was performed with anti-α-tubulin antibody. Quantification of the relative phosphorylated protein and α-tubulin optical density is given in arbitrary units (a.u.). (*G* and *H*) E14.5 cortical progenitors were obtained from $CB_1^{f/f}$ embryos, grown as neurospheres and nucleofected with pCAG-Cre-GFP or pCAG-GFP and cultured for 2 days in vitro (DIV). Immunofluorescence was performed with an anti-BrdU antibody (red) and $BrdU^+$ cells quantified in the electroporated GFP^+ cell population. Representative double-positive cells are pointed by empty arrowheads, while GFP-only positive cells are indicated by white arrows. (*I* and *J*) E14.5 cortical progenitors were obtained from $CB_1^{f/f}$ embryos, nucleofected with pCAG-Cre-GFP or pCAG-GFP and cultured for 2 DIV. Immunofluorescence with anti-pS6 (red) and anti-CB_1 (blue) antibodies and $pS6^+$ cells were quantified. Representative triple-positive cells are pointed by empty arrowheads, while GFP-only positive cells are indicated by white arrows.*$P < 0.05$; **$P < 0.01$ versus vehicle-treated cells and slices or control-nucleofected cortical progenitors. Scale bars: *A*, *C*, *G*, and *I*, 25 μm. Results correspond to 4 independent experiments.

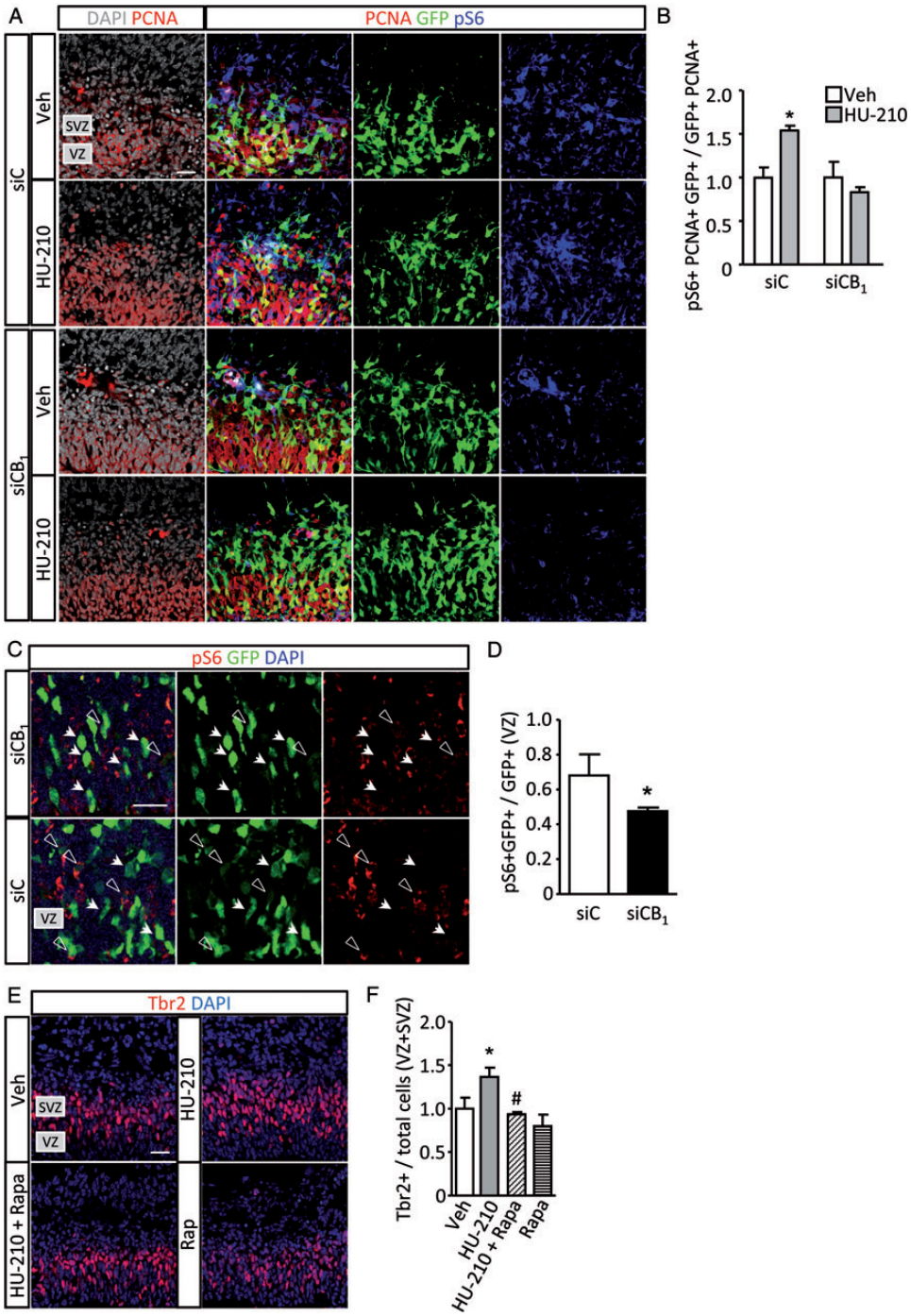

Figure 6. CB_1 cannabinoid receptor signaling regulates the generation of $Tbr2^+$ basal progenitors via mTORC1 signaling. (*A* and *B*) WT cortices were electroporated ex vivo at E14.5 with $siCB_1$ or siControl and pCAG-GFP, after 1 day in vitro (DIV), treated with HU-210 (1 μM) or vehicle. $pS6^+$ cells (blue) were quantified within the $PCNA^+$ (red) transfected (GFP^+) cell population after 2 DIV. Results are represented as the $pS6^+PCNA^+GFP^+$ cells/total $PCNA^+GFP^+$ cells normalized to the corresponding vehicle-treated slices. (*C* and *D*) In utero electroporation of siControl or $siCB_1$ and pCAG-GFP was performed at E13.5 and $pS6^+$ (red) cells were quantified at E16.5 in the ventricular zone (VZ) within the GFP^+ electroporated cell population. Empty arrowheads indicate double-positive cells, white arrows point GFP-only cells. $n=3$ embryos from at least 2 different litters per condition. (*E* and *F*) Cortical organotypic cultures from E14.5-WT embryonic brains were exposed for 48 h to vehicle or HU-210, alone or combined with rapamycin (1 μM). $Tbr2^+$ cells (red) were quantified and referred to total cell number in VZ/SVZ. Results are represented as the $Tbr2^+$ cell ratio normalized to vehicle-treated slices. Representative immunofluorescence images are shown. Scale bars: *A*, *C*, and *E*, 25 μm. Results correspond to 4 independent experiments. a.u., arbitrary units. $^*P < 0.05$; $^{**}P < 0.01$ versus vehicle-treated or control siRNA-electroporated slices.

CB_1 Cannabinoid Receptor Signaling Regulates the Pax6–Tbr2 Transcription Factor Axis During Cortical Progenitor Development

The Pax6–Tbr2 transcription factor axis is an essential regulator of the balance between self-renewal and neurogenesis, controlling the transition of radial glial to basal progenitors, and from proliferative progenitors to postmitotic neural cells (Hevner et al. 2001; Englund et al. 2005; Arnold et al. 2008; Sessa et al. 2008). Pax6 levels regulate radial glial cell cycle in a highly dose-dependent manner (Sansom et al. 2009). Pax6-

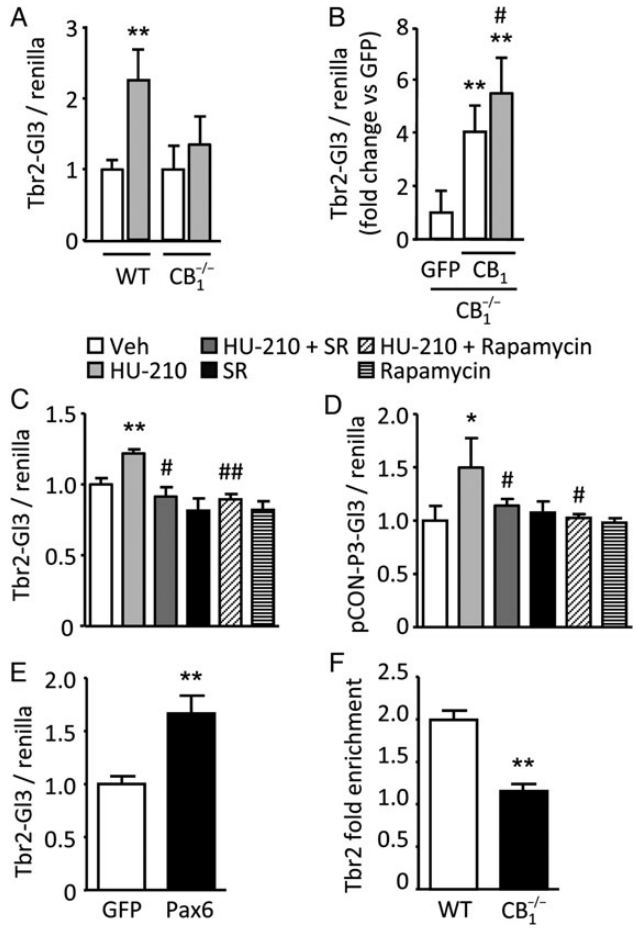

Figure 7. CB_1 receptor signaling regulates Tbr2 expression via Pax6 in an mTORC1-dependent manner. (*A*) WT and $CB_1^{-/-}$ cortical progenitors were transfected with a Tbr2-promoter activity reporter construct. After challenge with HU-210 (100 nM), Tbr2-promoter-driven firefly luciferase activity was determined and normalized with the constitutively expressed renilla luciferase. (*B*) $CB_1^{-/-}$ progenitors were transfected with pCAG-CB_1 48 h prior to HU-210 addition, and Tbr2-luciferase activity was quantified 24 h later. pCAG-GFP-transfected cells were employed as control. (*C*) Regulation of Tbr2-promoter-driven luciferase activity by HU-210 was analyzed in the presence of SR141716 (SR, 1 μM) or rapamycin (Rapa, 100 nM) in cortical progenitors nucleofected with the Tbr2-promoter luciferase construct. (*D*) CB_1 receptor activation regulates Pax6 transcriptional activity in an mTORC1-dependent manner, as determined after transfection with the Pax6 expression construct and the Pax6 consensus-binding sequences pCON/P3 reporter construct in the highly CB_1 receptor-expressing embryonic carcinoma P19 cells. Cells were treated and luciferase activity was determined as above. (*E*) Tbr2-promoter luciferase activity was quantified in cortical progenitor cultures after nucleofection of pCAG-Pax6 and pCAG-GFP. (*F*) Chromatin immunoprecipitation analysis was performed with an anti-Pax6 antibody and Tbr2-promoter DNA was quantified by qPCR in WT and $CB_1^{-/-}$ cortical extracts. Results correspond to 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle-treated cells, pCAG-GFP-transfected cells (*E*) or WT extracts (*F*); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus vehicle-treated pCAG-CB_1-transfected CB_1 knockout cells (*B*), or versus HU-210-treated cells (*C* and *D*).

mediated regulation of radial glial cell proliferation involves the control of symmetrical versus asymmetrical mode of cell division (Estivill-Torrus et al. 2002; Asami et al. 2011), and both gain and loss of function result in accelerated cell cycle exit and premature progenitor pool exhaustion (Quinn et al. 2007; Sansom et al. 2009). The complexity of the Pax6 role in regulating progenitor cell fate is highlighted by the diversity of cellular outcomes observed upon its manipulation, which can result in alterations of cell proliferation, cell cycle, and apoptosis (Osumi et al. 2008; Georgala et al. 2011). The puzzling pleiotropic actions of this transcription factor have been ascribed to a number of factors including, but not limited to, selectivity provided by different Pax6 subdomains, direct and indirect Pax6 targets, and context-dependent differences (Berger et al. 2007; Mi et al. 2013; Walcher et al. 2013). Likewise, Tbr2 deficiency results in defective SVZ cell proliferation and reduced neurogenesis, contributing to developmental alterations (Arnold et al. 2008; Sessa et al. 2008). Overall, altered activity of these transcription factors may not only compromise the appropriate number of neurons in the mature cortex, but also affect and restrict the diversity of neuronal identities acquired by their progeny (Franco et al. 2012). Consequently, alterations of different progenitor subpopulations contribute to cortical developmental disorders, including autism, Down syndrome and intellectual disability (Elsen et al. 2013; Tyler and Haydar 2013).

Results shown here demonstrate that CB_1 receptors are expressed, albeit at low levels, in the germinal zone of the mouse developing cortex. CB_1 receptors have been shown to be functional in cultures of neural progenitors from different origins, thus suggesting cell-autonomous actions in progenitor cells (Galve-Roperh et al. 2013). Nonetheless, the low expression levels of CB_1 receptors in the VZ/SVZ suggest that CB_1 receptors present in differentiated neurons may also contribute to the regulation of progenitor cell fate. Thus, the CB_1 receptor emerges as a novel signaling platform that coordinates progenitor cell expansion and neurogenesis by transducing information from the endocannabinoid tone present in neurogenic niches to endogenous neural identity determinants.

Pathophysiological Implications of CB_1 Cannabinoid Receptor-Mediated Regulation of mTORC1 Signaling

The endocannabinoid tone, via CB_1 receptors, regulates neural progenitor proliferation (Jin et al. 2004; Aguado et al. 2005; Mulder et al. 2008). However, the molecular mechanism of CB_1 receptor-mediated signaling in cortical progenitor expansion has remained elusive. Our new findings show that CB_1 receptor activation drives basal progenitor expansion and Tbr2 identity downstream of Pax6 induction by engaging the PI3K/Akt/mTORC1 signaling pathway. In agreement, CB_1 receptor-induced neurite outgrowth relies on the regulation of a transcription factor network that includes Pax6 activation via the PI3K pathway (Bromberg et al. 2008) and, in neurons, CB_1 receptor activates mTORC1 via PI3K/Akt (Puighermanal et al. 2009). In cerebellar progenitors, CB_1 receptor-induced cell proliferation is mediated by the PI3K/Akt pathway that, as a consequence of GSK3β inhibition, increases β-catenin nuclear translocation and cyclin D1 expression (Trazzi et al. 2010). Likewise, the CB_2 receptor, normally absent from mature neuronal cells but expressed in undifferentiated neural progenitors, promotes cell proliferation via PI3K/Akt/mTORC1 signaling (Palazuelos et al. 2012). In addition to the involvement of PI3K/Akt signaling in CB_1-mediated mTORC1 activation, a potential contribution of other pathways, such as the ERK cascade, is plausible.

mTORC1 signaling regulates neural cell fate, and a fine tuning of this signaling pathway is essential for appropriate cortical development (Crino 2011; Han and Sahin 2011). In the developing cortex, ablation of Raptor, one of the mTORC1 components, results in reduced cortical cell number and size, as well as microcephaly, a phenotype that, at least in part, is due to aberrant progenitor cell proliferation, cell cycle

alterations in the VZ/SVZ, and interference with apical $Sox2^+$ and basal $Tbr2^+$ progenitor cell populations (Cloetta et al. 2013). Our findings indicate that, in the absence of CB_1 signaling, mTORC1-driven Pax6 activity is impaired, and this may shift the mechanism of cell division toward neurogenesis (at the expense of the generation of intermediate progenitor cells), and leading to premature progenitor cell exhaustion. Noteworthy, mTORC1 activation in intermediate progenitor cells in adult neurogenic brain areas reverts progenitor quiescence in the aged brain (Paliouras et al. 2012). Thus, mTORC1 activation by CB_1 and likely also CB_2 cannabinoid receptors may contribute to injury-induced neural progenitor priming (Aguado et al. 2007; Palazuelos et al. 2012) and alleviate aging-associated decline of neurogenesis (Goncalves et al. 2008; Marchalant et al. 2009).

Alterations of intrinsic fate determinants that control neural progenitor identity, by either gain or loss of function, interfere with proper cortical development and ultimately have important consequences in neuronal excitability, cognition, and mood-anxiety disorders (Ramocki and Zoghbi 2008). Interference with Pax6 and Tbr2 expression exerts severe consequences on neurogenesis that, in turn, produce behavioral alterations (Sisodiya et al. 2001; Baala et al. 2007; Tuoc et al. 2009; Saito et al. 2011). Conditional cortical Pax6 knockout mice display strong behavioral deficits, including cognitive function and sensorimotor integration (Tuoc et al. 2009). Likewise, Tbr2-deficient mice show enhanced aggressiveness and hyperactivity (Baala et al. 2007; Arnold et al. 2008). Thus, deregulated activity of cell fate determinants can contribute to different neurodevelopmental disorders.

The instructive role of CB_1 receptor signaling in regulating the PI3K/Akt/mTORC1 pathway, and its impact on the Pax6–Tbr2 transcription factor code depicted here, point to a potential contribution of the endocannabinoid system to some of these neurodevelopmental disorders. Mutations of different upstream signaling elements of the mTORC1 pathway (e.g., tuberous sclerosis complex proteins Tsc1/Tsc2, Rheb1 and others) lead to the mTORC1 overactivation characteristic of tuberous sclerosis complex and focal cortical dysplasia patients (Crino 2011). While focal mTORC1 hyperactivation in the neocortex frequently results in intractable epilepsy, in the cerebellum, it may contribute to certain autism characteristics (Tsai et al. 2012).

CB_1 and CB_2 cannabinoid receptors have been shown to be expressed in developmental focal cortical alterations. In particular, increased CB_1 receptor expression was observed in type II focal cortical dysplasia lesions (Zurolo et al. 2010). CB_1 signaling may thus contribute to mTORC1 pathway overactivation and may interfere with the appropriate expression of progenitor identity determinants that is essential for progenitor expansion and coordination of cell cycle exit with radial migration (Kim et al. 2010; Orlova et al. 2010; Magri et al. 2011). Future studies are required to elucidate the possible contribution of CB_1 receptor signaling to the appearance of developmental pathologies characterized by an overactive mTORC1 signaling. In any case, understanding the role of the CB_1 receptor in cortical neurogenesis can contribute to increase our understanding of the behavioral consequences evoked by cannabinoid exposure of the developing brain on the predisposition to epilepsy and psychiatric disorders (Jutras-Aswad et al. 2009).

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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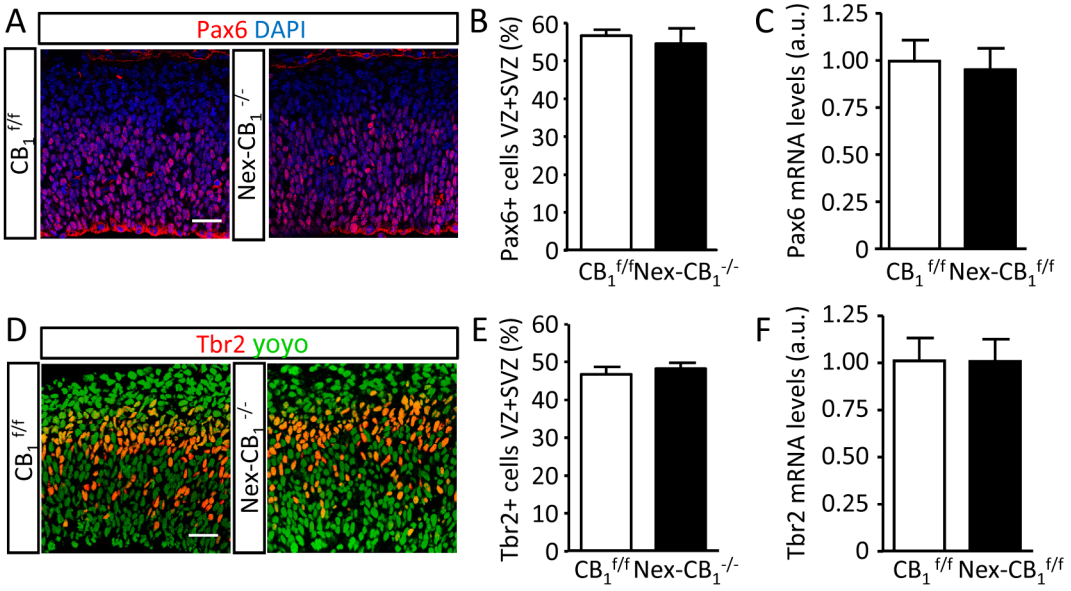
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Nex-CB1 -/-
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Tbr2+ cells VZ+SVZ (%)
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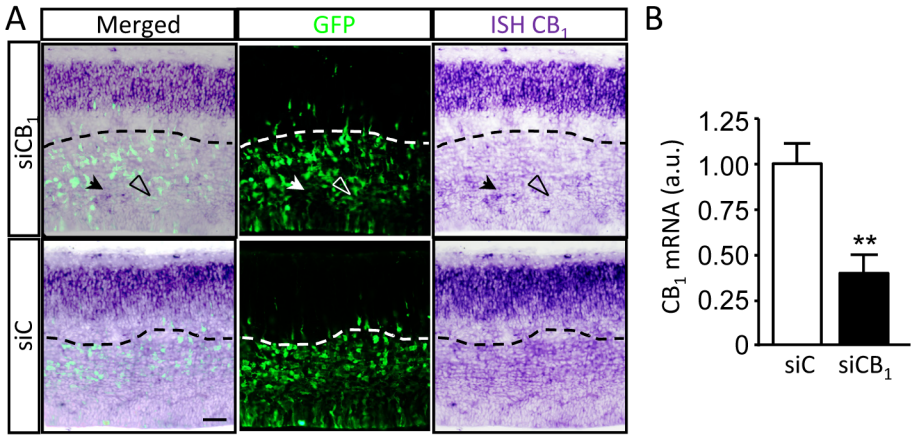
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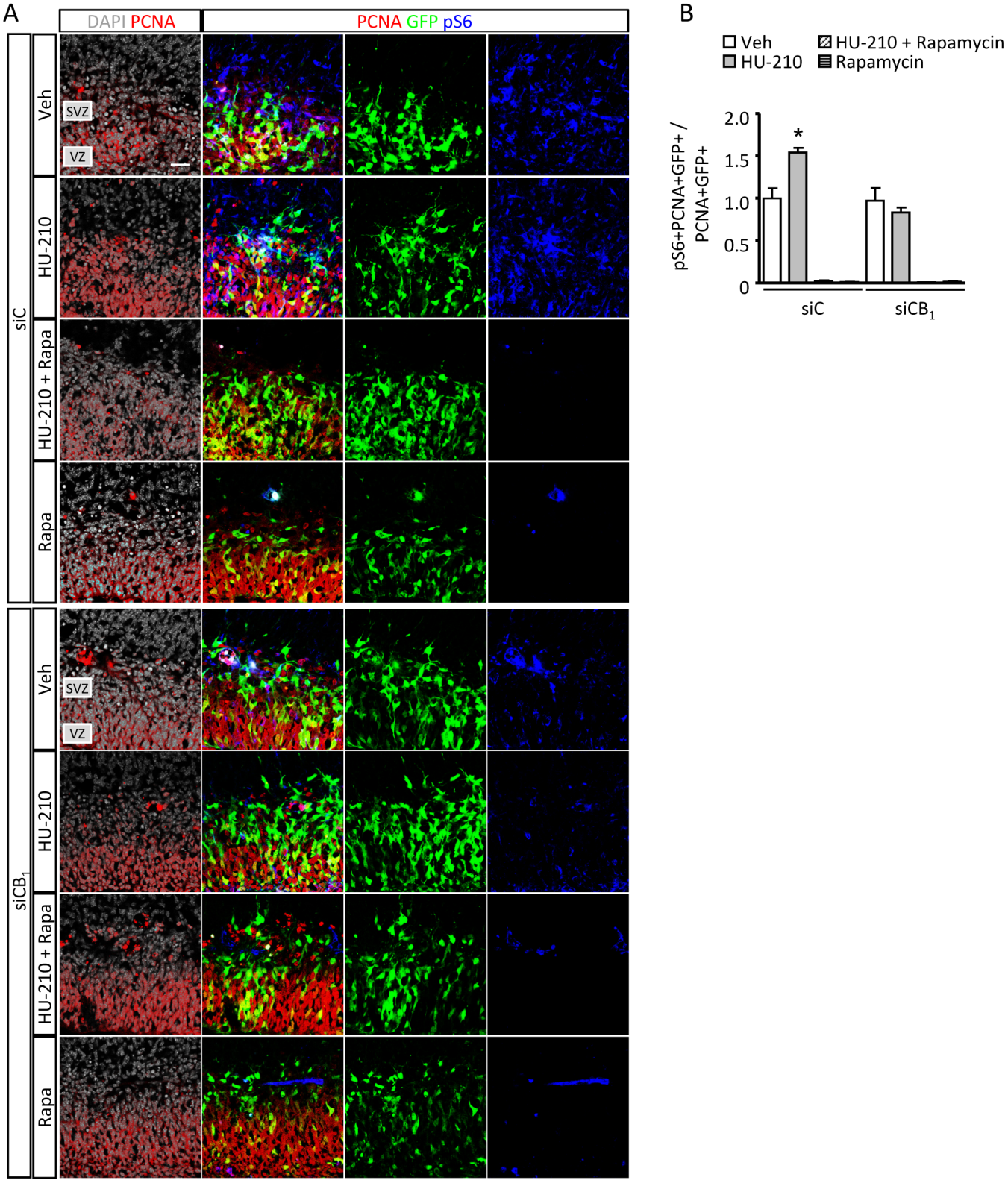
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3.2. **CHAPTER 2.** Role of CB_1 cannabinoid receptor signaling in corticospinal motor neuron development.

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Development/Plasticity/Repair

The CB_1 Cannabinoid Receptor Drives Corticospinal Motor Neuron Differentiation through the Ctip2/Satb2 Transcriptional Regulation Axis

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The generation and specification of pyramidal neuron subpopulations during development relies on a complex network of transcription factors. The CB_1 cannabinoid receptor is the major molecular target of endocannabinoids and marijuana active compounds. This receptor has been shown to influence neural progenitor proliferation and axonal growth, but its involvement in neuronal differentiation and the functional impact in the adulthood caused by altering its signaling during brain development are not known. Here we show that the CB_1 receptor, by preventing Satb2 (special AT-rich binding protein 2)-mediated repression, increased Ctip2 (COUP-TF interacting protein 2) promoter activity, and Ctip2-positive neuron generation. Unbalanced neurogenic fate determination found in complete $CB_1^{-/-}$ mice and in glutamatergic neuron-specific $Nex\text{–}CB_1^{-/-}$ mice induced overt alterations in corticospinal motor neuron generation and subcerebral connectivity, thereby resulting in an impairment of skilled motor function in adult mice. Likewise, genetic deletion of CB_1 receptors in *Thy1–YFP–H* mice elicited alterations in corticospinal tract development. Altogether, these data demonstrate that the CB_1 receptor contributes to the generation of deep-layer cortical neurons by coupling endocannabinoid signals from the neurogenic niche to the intrinsic proneurogenic Ctip2/Satb2 axis, thus influencing appropriate subcerebral projection neuron specification and corticospinal motor function in the adulthood.

Introduction

The development of the cerebral cortex involves the sequential formation and specification of the excitatory neuron populations that constitute the definitive six-layered cortical structure (Molyneaux et al., 2007; Fishell and Hanashima, 2008). Among the cell intrinsic mechanisms involved in corticogenesis, a complex proneurogenic transcription factor program is responsible for the correct establishment of neuronal identity within an area or layer (Guillemot et al., 2006; Molyneaux et al., 2007). These factors coordinate cell-cycle exit, neuronal migration, and the specific gene expression program that dictates neuronal identity of upper and deep cortical neurons and axonal connectivity. Neurons projecting to subcortical areas are primarily located in early-generated deep layers 5 and 6, whereas callosal projecting neurons are abundant in upper layers 2 through 4 (Sur and Rubenstein, 2005; Molyneaux et al., 2007). Specification of subcortical (i.e., corticothalamic and subcerebral) projection neurons relies on the combinatorial action of a transcriptional regulation network composed of different factors, such as special AT-rich sequence-binding protein 2 (Satb2), chicken ovoalbumin upstream promoter transcription factor I (COUP-TFI), and its interacting protein 2 (Ctip2/Bcl11b), a zinc finger transcriptional repressor (Arlotta et al., 2005; Alcamo et al., 2008; Tomassy et al., 2010). Forebrain embryonic zinc finger-like protein 2 (Fezf2) and its downstream regulator COUP-TF interacting protein 2 (Ctip2) are sufficient to induce ectopic specification of subcerebral projection neurons, and, in particular, of corticospinal motor neurons (CSMNs), which reside in layer 5b and extend their axons to the spinal cord (Molyneaux et al., 2005; Chen et al., 2008). In contrast, upper-layer specification factor Satb2 directly

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binds to matrix attachment regions (MARs) of the Ctip2 promoter and prevents Ctip2 expression, thus favoring the neuronal fate of callosal projection (Alcamo et al., 2008).

In addition to these endogenous determinants, an array of extracellular cues from the neurogenic niche is necessary for the precise coordination of cortical development. The endocannabinoid (eCB) system, via the cannabinoid CB_1 receptor, has been shown to exert a regulatory role in corticogenesis (Aguado et al., 2005; Berghuis et al., 2007; Morozov et al., 2009). Specifically, CB_1 receptor inactivation leads to defective ventricular/subventricular zone (VZ/SVZ) progenitor cell proliferation and axonal guidance alterations (Aguado et al., 2005; Mulder et al., 2008), thus impairing long-range corticothalamic connectivity (Mulder et al., 2008; Wu et al., 2010). However, the molecular mechanism of CB_1 receptor action in neuronal specification has not yet been investigated. Likewise, the impact on cortical development and the subsequent alterations in adult brain function as a consequence of altered prenatal CB_1 receptor function on intake of cannabinoid receptor agonists or antagonists by pregnant women remains primarily elusive (Galve-Roperh et al., 2009; Jutras-Aswad et al., 2009; Schneider, 2009). Hence, in the present study, we investigated the regulatory role of the CB_1 receptor in the transcription factor program that controls pyramidal neurogenesis and laminar differentiation, as well as its potential additional impact for adulthood neural functions. Our findings reveal that CB_1 receptor signaling, by modulating the Ctip2/Satb2 transcriptional regulatory code in differentiating neurons, controls neuronal projection fate differentiation, thereby tuning subsequent CSMN development and function.

Materials and Methods

Materials. The following materials were kindly donated: anti-CB_1 receptor antibody (K. Mackie, Indiana University, Bloomington, IN), $FAAH^{-/-}$ mice (B. Cravatt, Scripps Institute, San Diego, CA), pCAG–DsRed (M. Nieto, National Center of Biotechnology, Madrid, Spain), pfosluc constructs with the MAR sequences A2–A5 of the Ctip2 promoter and pMSCV–Satb2 expression vector (R. Grosschedl, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany), and HU-210 [(6a*R*,10a*R*)-3-(1,1′-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol] (R. Mechoulam, Hebrew University, Jerusalem, Israel). Rat monoclonal anti-5-bromo-2′-deoxyuridine (BrdU), rabbit polyclonal anti-Ctip2, anti-T-box brain 1 (Tbr1), anti-Tbr2, and mouse monoclonal anti-Satb2 antibodies were from Abcam. Rabbit polyclonal anti-CB_1 antibody (Frontier Institute, Hokkaido, Japan), mouse monoclonal anti-CRE (Covance), mouse monoclonal anti-GFP (Invitrogen), and chicken polyclonal anti-GFP (Millipore) antibodies were also used.

Animals. Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with Directive 86/609/EU of the European Commission. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Mice were maintained in standard conditions, keeping littermates grouped in breeding cages, at a constant temperature (20 ± 2°C) on a 12 h light/dark cycle with food and water *ad libitum*. The generation and genotyping of $CB_1^{-/-}$, $CB_1^{f/f,Nex\text{–}Cre}$, $CB_1^{f/f,Dlx5/6\text{-}Cre}$, and $FAAH^{-/-}$ mice and their respective littermate controls have been reported previously and was performed accordingly (Cravatt et al., 2001; Marsicano et al., 2003; Monory et al., 2007; Massa et al., 2010). Thy1–eYFP (line H) mice were obtained from The Jackson Laboratory [B6.Cg–Tg(Thy1–YFP–H)2Jrs/J] and crossed with $CB_1^{-/-}$ mice. The heterozygous F1 generation was crossed again with $CB_1^{-/-}$ mice. Mouse tissues of either sex were obtained during timed mating as assessed by vaginal plug.

Immunofluorescence and confocal microscopy. Coronal brain slices (10 μm) were processed as described previously (Mulder et al., 2008), and layers were identified by their discrete cell densities as visualized by Hoechst 33528 (Sigma) and β-III-tubulin counterstaining. After blockade with 5% goat serum, brain sections were incubated overnight at 4°C with the indicated primary antibodies. Confocal fluorescence images were obtained in a blinded manner by an independent observer, and all quantifications were obtained from a minimum of six sections from 1-in-10 series per mice. Immunofluorescence of cortical sections was performed along the rostral-to-caudal axis, and the quantifications were performed in the mediolateral area of rostromedial sections that correspond to the motor/somatosensory cortex. Pyramidal layer specification was determined at embryonic day 16.5 (E16.5), postnatal day 2 (P2), and P8 in a 50-μm-wide cortical column divided into 10 equally sized bins, from the ventricular surface to the marginal zone. At least two independent cortical columns were analyzed per section, and results were averaged (see Figs. 1, 2, 6, 8). Positive cells for the corresponding markers were quantified and referred to the total cell number in the bin identified by Hoechst 33528.

Protein kinase Cγ immunohistochemistry. Protein kinase Cγ (PKCγ) immunohistochemistry was performed in 100-μm-thick sagittal sections. After permeabilization, quenching of endogenous peroxidase activities and blockade with goat serum free-floating sections were incubated with rabbit anti-PKCγ antibody (Santa Cruz Biotechnology) and processed according to the instructions of the manufacturer with 1:500 goat biotinylated anti-rabbit IgG and with avidin-biotinylated peroxidase complex (ABC Standard Vectastain ABC kit; Vector Laboratories). Stained sections were mounted, and bright-field images were captured from an Olympus BX51 upright microscope using an Olympus DP70 CCD camera.

In situ *hybridization.* Coronal paraffin sections (10 μm) of E12.5, E13.5, E14.5, and E16.5 heads were obtained, deparaffinized, and processed for *in situ* hybridization as described previously (Monory et al., 2007). Clim1 (National Center for Biotechnology Information reference sequence NM_ 010698.3) riboprobe for *in situ* hybridization was amplified with the following primers: forward, ACCCTCATTCCCCGTTATT; and reverse, TGGCTCTCCTACCACCATC.

Real-time quantitative PCR. RNA was isolated using RNeasy Plus kit (Qiagen). cDNA was obtained with Transcriptor (Roche). Real-time quantitative PCR assays were performed using the FastStart master mix with Rox (Roche), and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA and β-actin levels as reference.

Ex vivo *and* in utero *electroporation. Ex vivo* electroporation experiments were performed at E13.5 as described previously (Mulder et al., 2008) with pCAG–GFP and pCAG–CB_1–GFP expression vectors, or pGFP-V-RS (Origene) shControl and shCB_1 and dissociated cells were cultured as described below. In addition, $CB_1^{f/f}$ cortices were electroporated with pCAG–GFP or pCAG–Cre–GFP (Addgene). In addition, *in utero* electroporations were performed at E14.5 or E13.5 with pCAG–CB_1–GFP or DsRed (respectively) and analyzed at E16.5 (see Figs. 1*I*,*J*, 7*A*–*D*). *In utero* electroporation experiments with pCAG–CRE–GFP and pCAG-GFP control plasmids were also performed from E12.5 to P0 in $CB_1^{f/f}$ mice (see Fig. 4*D*,*E*).

Pyramidal and organotypical cortical cell cultures. Cortical neural progenitors were cultured from dissected cortices isolated at E13.5. Cells were mechanically dissociated and plated in polylysine- and laminin-coated dishes after *ex utero* electroporation and dissection of the cortical electroporated area (fast green positive). The neuronal phenotype was assessed after 7 d *in vitro* by quantification of Ctip2 and Satb2 expression in GFP-positive (GFP^+) cells from ≥10 randomly selected view fields/coverslip after Hoechst 33528 cell counterstaining in genetically manipulated cells. In addition, pharmacological regulation experiments were performed in wild-type (WT) brain slices.

Gene promoter activity assays. The neural stem cell line HiB5 was used after transient transfection with 0.75 μg of the A4- or A3–MAR–pfosluc reporter of the Ctip2 promoter, 0.5 μg of pCAG–CB_1–GFP, 0.25 μg of pMSCV–Satb2, and 0.02 μg of renilla-derived luciferase as internal transfection control with Lipofectamine 2000. Transcriptional promoter-driven luciferase activity was performed using the Dual-Luciferase Reporter Promega assay system and renilla-derived luciferase activity as internal transfection control in a Lumat LB9507 luminometer (Berthold Technologies). Control experi-

ments were performed with an excess of Satb2 expression that was able to efficiently block the Ctip2-reporter activity (data not shown).

Behavioral analyses. $CB_1^{-/-}$ and *Nex–*$CB_1^{-/-}$ mice and their respective wild-type littermates (WT and $CB_1^{f/f}$) were analyzed at 8 weeks of age with paired mean age among groups. Animals were always tested during the same light phase and acclimatized to the testing room for at least 30 min. All tests were video recorded for subsequent analysis and double-blind quantification. Mice were food deprived the night before the testing day (mean body weight at the end of the test becoming 92.6 ± 2.2% of the initial body weight). Skilled motor test and patch removal tasks were performed according to established protocols (Tomassy et al., 2010). Briefly, a Plexiglas reaching box (20 cm long × 8 cm wide × 20 cm high), with a 1-cm-wide vertical slit in the front side of the box was used. Animals had to reach the palatable food pellet (20 mg dustless precision sucrose-flavored food pellets (Bio Serv) from a shelf (4 cm wide × 8 cm long) in front of the vertical slit. Mice were habituated to the sucrose-flavored pellets for 3 consecutive days before the tests, which consisted of three phases: habituation, unskilled reaching, and skilled reaching. In the habituation phase (two sessions), mice were placed in the testing cage and 10 pellets were scattered on the floor. The session finished when mice had eaten all the pellets or 10 min had passed. For the unskilled reaching test, food pellets were placed one by one on the shelf within the mouse's tongue-reaching distance. The test finished when 10 pellets were eaten or 4 min had passed. In the skilled reaching test, food pellets were placed one by one on the shelf 1.5 cm away from the slit, so that mice had to use their forelimbs to reach them. Pellet grasping and retrieval was scored as a success, and pellet displacement without retrieval was scored as an error. The test finished within 6 min. Results are represented as percentage of success [(total successes/total trials) × 100]. The absence of phenotypic alterations in $CB_1^{-/-}$ and *Nex–*$CB_1^{-/-}$ mice in the unskilled task was used as a control that CB_1 receptor ablation per se does not interfere with the test by influencing factors different from corticospinal function. Complementary analysis of motor impairment in $CB_1^{-/-}$ mice was conducted by using the staircase reaching test (Campden Instruments). This test allows measurement of coordinated paw reaching in rodents. The system is formed by two stairs with eight steps each on which two rewarding food pellets can be placed. The test consisted of three phases: training, unskilled reaching, and skilled reaching. Habituation to sucrose-flavored food pellets was performed for 3 consecutive days, and then mice were habituated to the apparatus for 2 d by placing some pellets along the central corridor and stairs. On 5 consecutive training days, both stairs were filled with two pellets per stair, and mice were challenged to reach pellets that had been placed exclusively in the stairs for 10 min. In two additional test sessions, animals were challenged to reach pellets placed in the five lowest steps, which need the use of a paw to be reached (note that the three upper steps can be reached with the tongue and thus are not useful to assess paw skilled reaching ability). Finally, the patch removal test was conducted to measure skilled motor performance by assessing the ability of the mouse to remove a piece of adhesive patch placed in each hindpaw as described previously (Tomassy et al., 2010). Adhesive patches were placed in each hindpaw of the mouse, and the animal was placed in a normal housing cage and video recorded. The test finished when both patches had been removed or 4 min had passed. The time of latency for the first nose contact with the patch was determined, thus providing a general assessment of the sensorial status. Skilled patch removal ability was measured by calculating the mean number of contacts until each adhesive patch had been removed. Results shown correspond to the average of two tests. Additional characterization of general motor activity, exploration, and coordination was performed in ActiTrack and RotaRod devices as described previously (Blazquez et al., 2011).

DiI labeling. Labeling of corticospinal tracts with the lipophilic carbocyanine dye DiI (1,1′-diotadecyl-3,3,3′,3′-tetramethylindocarbocyanine; Invitrogen) was performed in the brains of *Nex–*$CB_1^{-/-}$ and $CB_1^{f/f}$ mice at P2. A single crystal of DiI was picked up on a fire-polished tip of a broken glass micropipette and inserted into the presumptive motor cortex. The DiI-containing brains were incubated in PBS/0.1% sodium azide at 50°C in the dark for 4 weeks. Brains embedded in 3% agar were sectioned at 100 μm in the sagittal plane, counterstained with DAPI, and mounted with PBS. Axon projections were visualized using a Carl Zeiss AxioImager M1 with 5× magnification and 0.16 numerical aperture Zeiss objectives. Confocal images were obtained using a Carl Zeiss 510 system with 10×/0.45 numerical aperture (air) objective lens.

Data analyses and statistics. Results shown represent the means ± SEM, and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way ANOVA, as appropriate. A *post hoc* analysis was made by the Student–Neuman–Keuls test.

Results

The CB_1 cannabinoid receptor regulates cortical layer neuron specification

We initially determined the impact of CB_1 receptor inactivation on cortical development by analyzing cortical thickness of $CB_1^{-/-}$ and WT littermates. $CB_1^{-/-}$ mice showed enlarged ventricles at P2, in agreement with the described alterations induced by *in utero* CB_1 receptor pharmacological blockade (Mulder et al., 2008). A reduction of total cortical thickness was evident at P2 in $CB_1^{-/-}$ mice (total cortical thickness, 527 ± 26 μm in WT mice vs 475 ± 15 μm in $CB_1^{-/-}$ mice; $p < 0.05$), which was attributable to deep-layer but not upper-layer thickness reduction (upper- and deep-layer cortical thickness, 174 ± 26 and 352 ± 28 μm in WT mice vs 172 ± 5 and 302 ± 20 μm in $CB_1^{-/-}$ mice; $p < 0.05$ for deep-layer cortical thickness; $n = 6$ for each group). These results support a significant role of the CB_1 receptor in controlling progenitor population size and raise the question of whether CB_1 receptor signaling exerts a selective function in the differentiation of the neuronal populations of the different cortical layers. Because Tbr1 is an early expressed T-box transcription factor that promotes deep-layer specification and corticothalamic neuron projections (Hevner et al., 2001; Han et al., 2011), we first analyzed postmitotic $Tbr1^+$ neuroblasts along the developing cortex in CB_1 receptor-deficient mice. Quantification of $Tbr1^+$ cells in equal-sized bins at E16.5 showed that these postmitotic cells accumulated abnormally in deep bins of the cortical plate of $CB_1^{-/-}$ mice compared with WT littermates (Fig. 1*A*, middle column, *B*). Moreover, the distribution of neurons that express the upper-layer neuronal marker Satb2 was also affected (Fig. 1*A*, right column), and, in particular, $Satb2^+$ cells were expanded in the lower bins 2–3 (Fig. 1*C*). Double marker analysis further showed that CB_1 receptor deletion increased the number of cells that coexpress Tbr1 and Satb2 (Fig. 1*D*,*E*). Satb2 is a well-known repressor of Ctip2, a selective layer 5b marker of subcerebral projection neurons, and this repressive action of Satb2 promotes callosal projection-neuron identity (Alcamo et al., 2008). We sought to investigate the impact of CB_1 deletion in the development of $Satb2^+$ and $Ctip2^+$ neuronal cell populations. $Satb2^+$ cells in $CB_1^{-/-}$ cortices at E16.5 were intermingled among $Ctip2^+$ cells, and an increased number of cells coexpressing both markers were evident during CB_1 receptor inactivation (Fig. 1*D*,*F*). $Satb2^+$ cell number in the total cortical column was significantly increased (Fig. 1*G*), and consequently $Ctip2^+$ cells were reduced in $CB_1^{-/-}$ mice compared with WT littermates (Fig. 1*H*).

Considering the altered expression of neuronal specification markers induced by genetic ablation of CB_1, we performed gene expression analysis of selected transcription factors involved in the regulation of corticogenesis in E14.5 cortical extracts. CB_1 receptor inactivation impaired the expression of determinants known to be involved in fate specification and development of deep- and upper-layer cortical neurons. Thus, CB_1 receptor deficiency decreased the expression of the deep-layer neuronal markers Fezf2 and Ctip2 (relative mRNA levels, 0.55 ± 0.07 vs 1.00 ± 0.15 in WT cortices and 0.47 ± 0.06 vs 1.00 ± 0.13 in WT

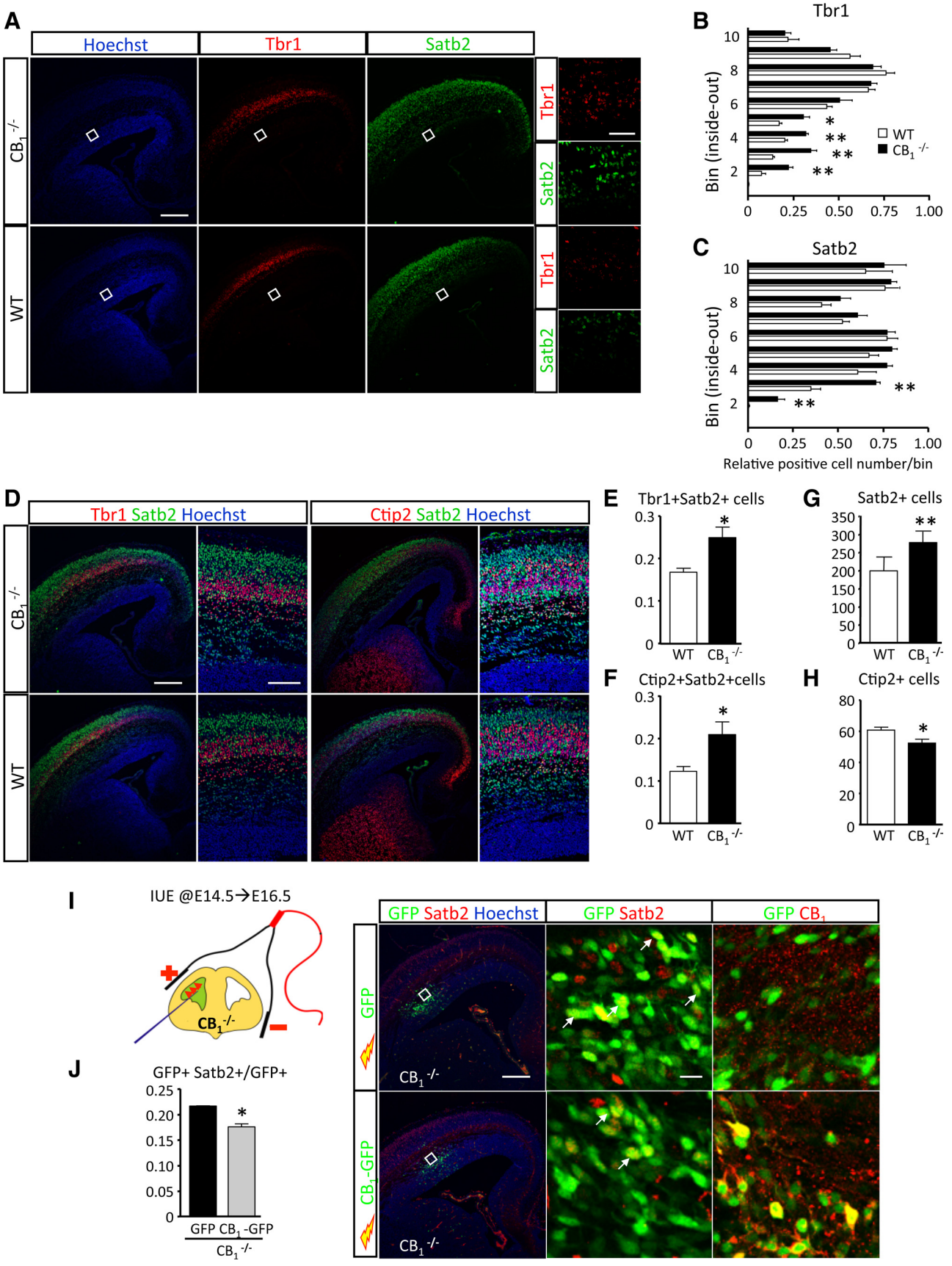

Figure 1. CB_1 receptor inactivation interferes with the specification of upper- and deep-layer cortical neurons. ***A***, $CB_1^{-/-}$ mice and WT littermates were analyzed at E16.5, and $Tbr1^+$ and $Satb2^+$ cells were revealed by immunofluorescence. Low- and high-magnification representative images are shown. ***B***, ***C***, The fraction of differentiating cells that express Tbr1 and Satb2 in $CB_1^{-/-}$ and WT mice (black and white bars, respectively) was quantified in equal-size binned areas and referred to total cell number (Hoechst 33528 counterstaining). ***D***, Immunofluorescence analysis was performed in WT and $CB_1^{-/-}$ mice at E16.5 for $Tbr1^+$, $Ctip2^+$, and $Satb2^+$ cells. Representative images of Tbr1–Satb2 and Ctip2–Satb2 immunoreactivity are shown (*Figure legend continues.*)

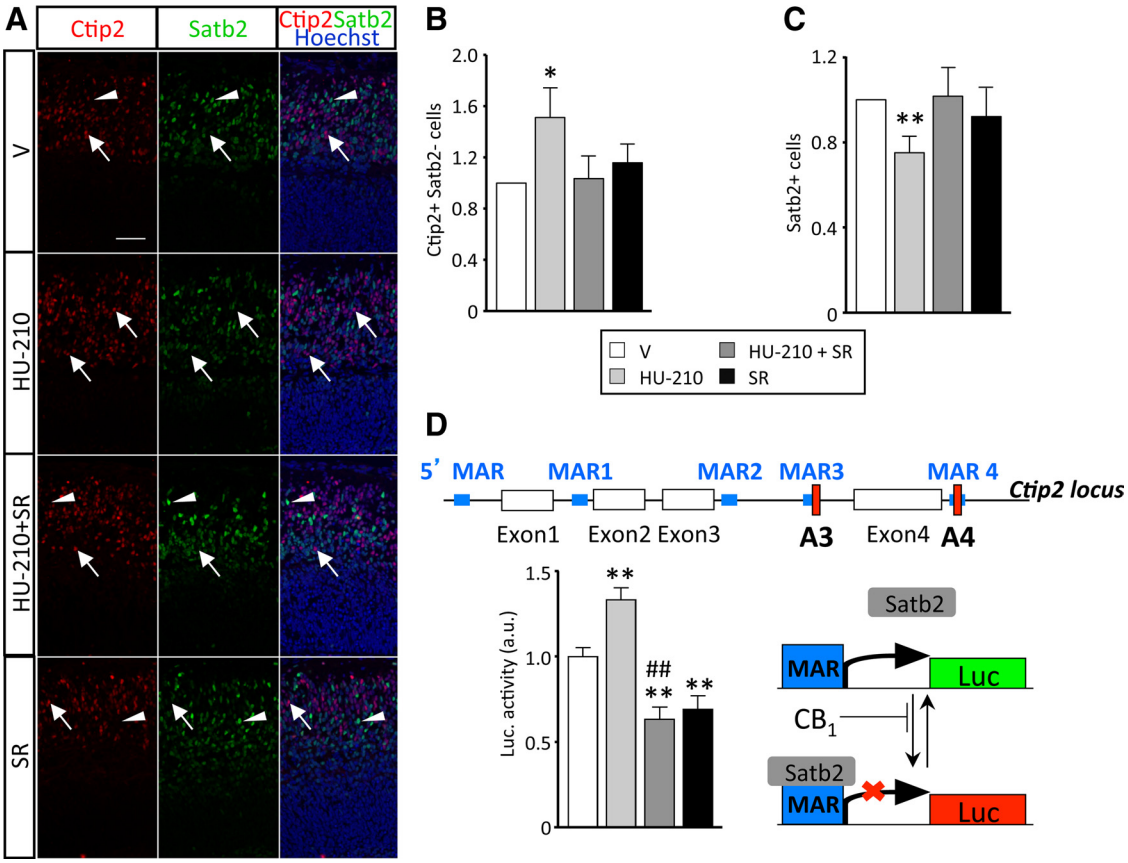

Figure 2. CB_1 receptor signaling promotes $Ctip2^+$ neuron differentiation. ***A***, Cortical organotypic cultures form E13.5 WT embryos were treated for 3 d with vehicle (V) or HU-210 (5 μM), alone or combined with SR141716 (25 μM). Representative immunofluorescence images of $Ctip2^+$ and $Satb2^+$ cells are shown (red and green, respectively). ***B***, ***C***, The fraction of Ctip2-only positive cells (arrows) and $Satb2^+$ cells (arrowheads) were quantified in the same conditions and referred to total cell number (Hoechst 33528 counterstaining). Results correspond to four independent experiments. ***D***, HiB5 neural stem cells were transfected with a luciferase reporter construct that contained the A4 MAR sequence of the Ctip2 locus together with pCAG–CB_1 and pMSCV–Satb2. Ctip2-luciferase activity was determined 36 h after treatment with vehicle (V) or HU-210 (50 nM), alone or combined with SR141716 (1 μM). $^*p < 0.05$, $^{**}p < 0.01$ versus vehicle-treated cells; $^{\#\#}p < 0.01$ versus HU-210-treated cells. Scale bar: ***A***, 50 μm.

cortices; $p < 0.05$ and $p < 0.01$, respectively; $n = 4$ for each group) (Arlotta et al., 2005; Chen et al., 2008) and the corticofugal neuronal marker Sox5 (relative mRNA levels, 0.50 ± 0.05 vs 1.00 ± 0.11 in WT cortices; $p < 0.05$) (Lai et al., 2008). Conversely, levels of the upper-layer transcription factor Satb2 were upregulated in $CB_1^{-/-}$ cortices (relative mRNA levels, 2.65 ± 0.15 vs 1.00 ± 0.23 in WT cortices; $p < 0.05$). To validate the role of the CB_1 receptor in the correct expression of upper/deep-layer neuronal specification markers, we performed a gain-of-function strategy aimed at rescuing CB_1 receptor expression in a $CB_1^{-/-}$ background by *in utero* electroporation of a pCAG–CB_1–GFP expression vector (Fig. 1*I*). Importantly, reexpression of the CB_1 receptor reduced the expansion of $Satb2^+$ cells that occurred in deep bins of $CB_1^{-/-}$ cortices and thus reduced the number of GFP^+Satb2^+ cells (Fig. 1*I*, arrows, *J*).

The CB_1 cannabinoid receptor controls deep-layer neuron specification through the regulation of Ctip2–Satb2 balance

We further evaluated the role of the CB_1 receptor in the regulation of deep-layer neuronal specification by analyzing the changes in the Ctip2–Satb2 axis in cortical organotypic cultures of E13.5 WT mice. Treatment with HU-210, a CB_1 receptor synthetic agonist, increased the number of Ctip2-only positive cells but decreased the number of $Satb2^+$ cells (Fig. 2*A–C*, arrows and arrowheads, respectively). The induction of neuronal differentiation to $Ctip2^+$ cells by HU-210 and its inhibitory effect on $Satb2^+$ cell differentiation were prevented by the CB_1 receptor-selective antagonist SR141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide]. Because Satb2 negatively regulates Ctip2 expression by binding to MAR sequences at the Ctip2 promoter (Alcamo et al., 2008; Britanova et al., 2008), we performed luciferase reporter assays for different MAR regions of the Ctip2 promoter in the HiB5 neural stem cell line. HiB5 cells were transiently transfected with luciferase constructs under the control of the Satb2-binding sites A4 or A3 of the Ctip2 promoter together with expression vectors for CB_1 and Satb2. HU-210 treatment increased A4–fosluc activity (Fig. 2*D*), and this effect was prevented by CB_1 receptor blockade with SR141716. Likewise, HU-210 increased A3–fosluc activity (data not

←

(*Figure legend continued.*) (left and right panels, respectively). ***E***, ***F***, Quantification of the neuronal cell fraction that coexpress Tbr1 with Satb2 and Satb2 with Ctip2, respectively, referred to total cell number (Hoechst 33528 counterstaining) in the cortical column of WT and $CB_1^{-/-}$ mice (white and black columns, respectively). ***G***, ***H***, $Satb2^+$ and $Ctip2^+$ cells were quantified in 50-μm-wide cortical columns of the same mice. ***I***, ***J***, *In utero* electroporation (IUE) of E14.5 $CB_1^{-/-}$ mice was performed with pCAG–GFP vector and pCAG–CB_1–GFP to reexpress CB_1 receptor (black and gray bars, respectively), and cortices were subsequently analyzed at E16.5. $Satb2^+$ cells in the GFP^+ electroporated cell population were quantified. Arrows indicate electroporated double GFP^+Satb2^+ cells. $n = 5$ for each group in ***A–G*** and $n = 2$ for each group in ***H*** and ***I***. Scale bars: ***A***, 250 μm and inset, 35 μm; ***D***, 250 and 100 μm; ***I***, 250 μm and inset, 20 μm. $^*p < 0.05$, $^{**}p < 0.01$ versus control mice.

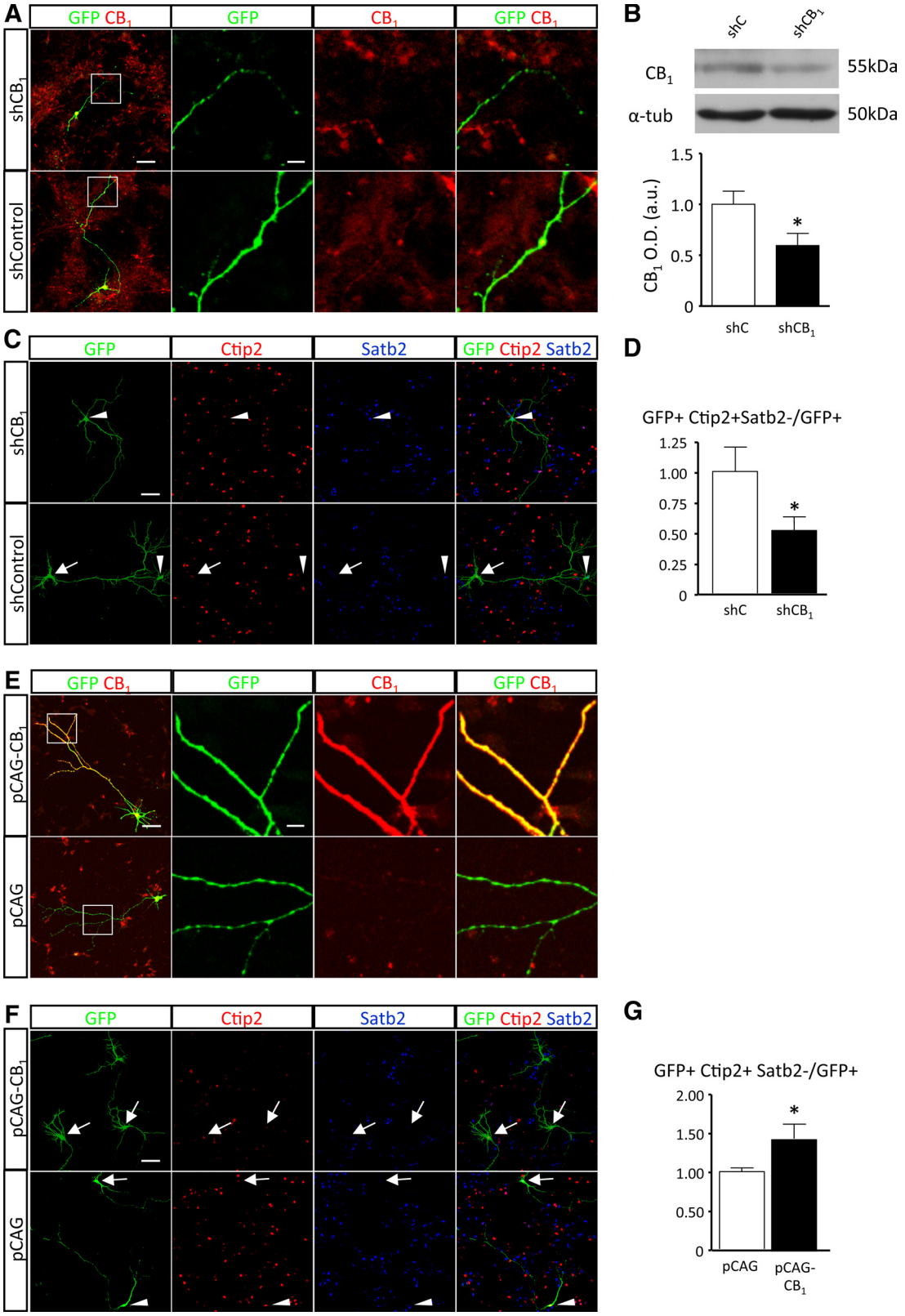

Figure 3. Manipulation of CB_1 receptor expression controls Ctip2$^+$ neuron differentiation. ***A***, Primary cortical cultures obtained from E13.5 WT cortices after *ex utero* electroporation with shControl and $shCB_1$ were allowed to differentiate for 7 d *in vitro* (DIV). Low- and high-magnification images are shown of CB_1 receptor expression (red) in electroporated cells identified with GFP antibody (green). ***B***, Western blot analysis of CB_1 receptor knockdown after transfection of shControl (shC) and $shCB_1$ in P19 cells. α-tub, α-Tubulin; O.D., optical density; a.u., arbitrary units. ***C***, ***D***, Ctip2-only-positive cells (arrow) and Satb2$^+$ cells (arrowheads) were quantified in the electroporated GFP$^+$ cell subpopulation. Representative immunofluorescence images are shown. ***E***, Primary cortical cultures of E13.5 WT cortices after *ex utero* electroporation with pCAG and pCAG–CB_1 and differentiation 7 DIV. Low- and high-magnification images are shown of CB_1 receptor expression in electroporated cells identified with GFP antibody. ***F***, ***G***, Ctip2-only-positive cells (arrows) and Satb2$^+$ cells (arrowhead) were quantified in the electroporated GFP$^+$ cell subpopulation. Representative immunofluorescence images are shown. Scale bars: ***A***, 50 and 10 μm; ***C***, ***F***, 50 μm; ***E***, 50 and 10 μm. *$p < 0.05$ versus control-electroporated cells. Results correspond to four independent experiments.

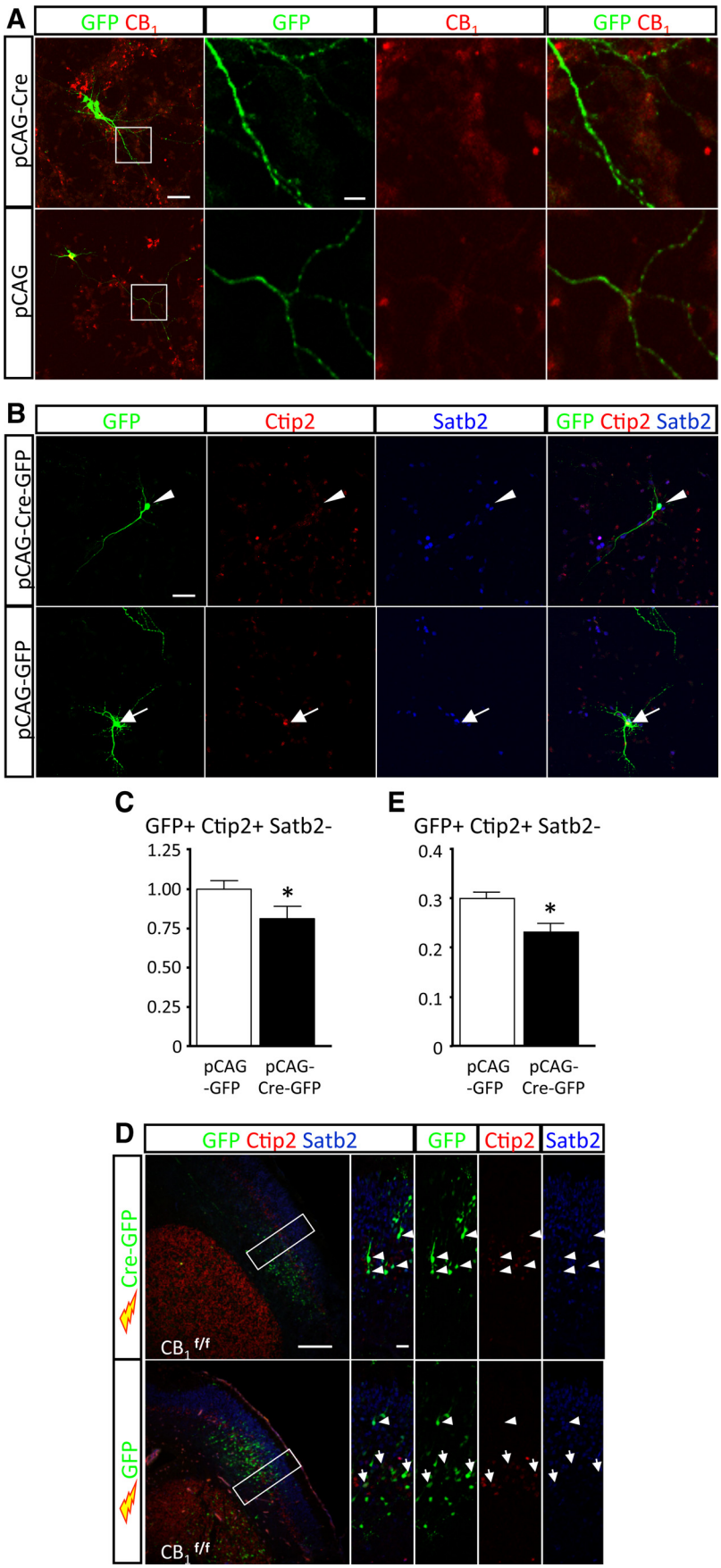

Figure 4. ***A***, CB_1 receptor expression was analyzed in electroporated cells (GFP^+) in $CB_1^{f/f}$-derived cells after pCAG–GFP or pCAG–CRE–GFP electroporation of $CB_1^{f/f}$ cortices. ***B***, ***C***, Ctip2-only-positive cells (arrow) and $Satb2^+$ cells (arrowhead) were quantified in the electroporated GFP^+ cell subpopulation. Representative immunofluorescence images are shown. Results correspond to four independent experiments. ***D***, ***E***, *In utero* electroporation of E12.5 $CB_1^{f/f}$ mice was performed with pCAG–GFP and pCAG–CRE–GFP vectors, and pups were subsequently analyzed at P0. Ctip2-only-positive cells that were electroporated ($Ctip2^+Satb2^-GFP^+$) were quantified, and the relative number to the GFP^+ cell population is shown. Arrows indicate electroporated $Ctip2^+Satb2^-GFP^+$ cells (arrowheads indicate $Satb2^+GFP^+$ cells). $n = 3$ for each group. $^*p < 0.05$ versus control electroporated cells or pups. Scale bars: ***A***, 50 and 10 μm; ***B***, 50 μm; ***D***, 250 and 25 μm.

shown). Thus, CB_1 receptor activity prevents the repressive effect of Satb2 on the Ctip2 promoter, thereby increasing its activity.

To directly assess the role of the CB_1 receptor in deep-layer neuronal differentiation, we electroporated E13.5 mouse cortices, when CSMN generation is maximal (Tomassy et al., 2010), with a GFP^+ vector, and the CB_1 receptor was knocked down or overexpressed with $shCB_1$ and pCAG–CB_1, respectively. Cortical cultures were obtained by dissociation and allowed to differentiate for 7 d *in vitro*. Efficient manipulation of CB_1 receptor expression under these conditions was evaluated by CB_1 and GFP immunofluorescence (Fig. 3*A*,*E*). In addition, we validated the effect of $shCB_1$ by Western blot analysis (Fig. 3*B*) and real-time PCR. $shCB_1$ reduced CB_1 protein and mRNA levels when compared with shControl (relative CB_1 protein levels in shControl and $shCB_1$, 1.00 ± 0.13 and 0.59 ± 0.15, $p < 0.01$; relative CB_1 mRNA levels in shControl and $shCB_1$, 1.00 ± 0.23 and 0.46 ± 0.17, $p < 0.01$). Immunofluorescence characterization of neuronal populations among the electroporated GFP^+ population after differentiation revealed that CB_1 receptor downregulation decreased the generation of Ctip2-only-positive cells (Fig. 3*C*,*D*), whereas CB_1 receptor overexpression increased $Ctip2^+$ cells (Fig. 3*F*,*G*). In addition, CB_1 ablation by *ex utero* electroporation of pregnant E13.5 $CB_1^{f/f}$ brains was conducted with a Cre recombinase expression vector (Fig. 4*A*). Using this strategy to achieve acute CB_1 receptor loss of function, we also observed reduced $Ctip2^+$ differentiation of cortical cells when compared with GFP^+ cells (Fig. 4*B*, arrows, *C*). To further validate the results derived from *ex utero* CB_1 receptor manipulation, we performed *in vivo in utero* electroporation with the pCAG–Cre–GFP and pCAG–GFP plasmids, and pups were analyzed at P0 (Fig. 4*D*). Noteworthy, *in*

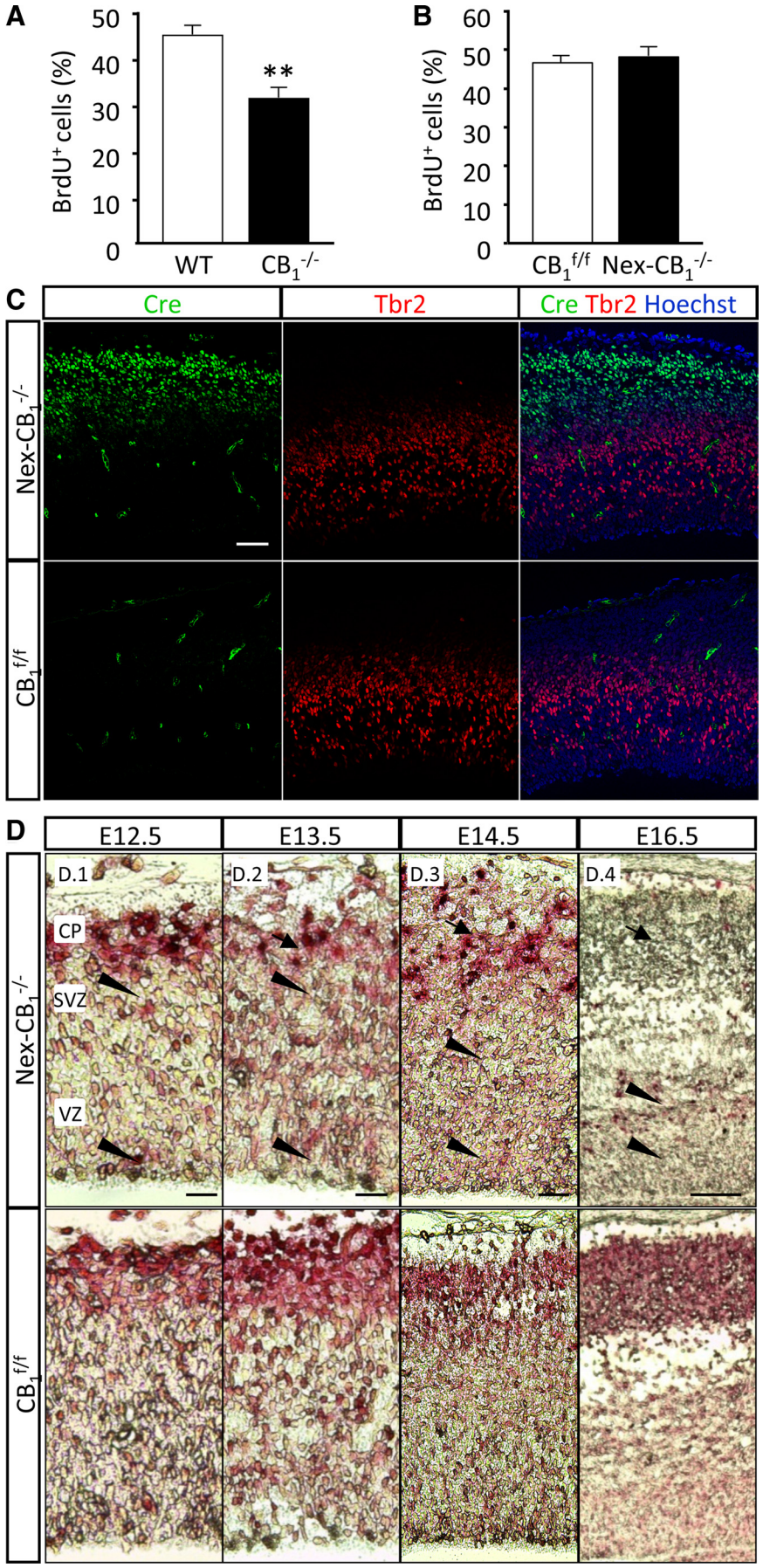

Figure 5. Cortical progenitor cell proliferation is not affected in conditional *Nex–$CB_1^{-/-}$* mice. ***A–B***, Quantification of progenitor cell proliferation as BrdU-labeled cells in the VZ/SVZ of *$CB_1^{-/-}$* mice and WT littermates, and *Nex–$CB_1^{-/-}$* mice and *$CB_1^{f/f}$* littermates, at E14.5. ***C***, Cre recombinase expression in the same animals as determined by immunofluorescence analysis of Cre and Tbr2 (green and red, respectively). ***D***, *In situ* hybridization of CB_1 mRNA in *Nex–$CB_1^{-/-}$* mice and *$CB_1^{f/f}$* littermates at E12.5, E13.5, E14.5, and E16.5 (***D.1–D.4***, respectively). Arrows indicate CB1 mRNA reduced expression in postmitotic cortical areas; arrowheads indicate preserved expression in VZ/SVZ. Scale bars: ***C***, ***D.3***, 50 μm; ***D.1***, ***D.2***, 30 μm; ***D.4***, 100 μm.

utero CB_1 receptor ablation decreased $Ctip2^+$ neuronal differentiation when compared with control *$CB_1^{f/f}$* cortices (Fig. 4*E*). These results support that CB_1 receptor activity is required for the appropriate expression of the deep-layer neuronal determinant Ctip2.

The CB_1 cannabinoid receptor independently regulates progenitor proliferation and neuronal differentiation

Because CB_1 receptor loss of function is ensued by alterations in neuronal specification during cortical development (present study) and the CB_1 receptor is also known to be expressed in progenitor cells, on which it drives VZ/SVZ progenitor proliferation in a cell-autonomous manner (Aguado et al., 2005; Mulder et al., 2008), a plausible hypothesis would be that CB_1-receptor-mediated regulation of neuronal specification was a direct consequence of its role on progenitor generation. To investigate the putative relationship between changes in cortical progenitor proliferation and neuronal specification, we next compared complete *$CB_1^{-/-}$* mutant mice and glutamatergic-specific *Nex–$CB_1^{-/-}$* mutant mice. In the latter mutants, CB_1 is selectively deleted in glutamatergic neurons of the dorsal telencephalon (Monory et al., 2006). Cre recombinase expression under the control of the Nex regulatory sequences was described to selectively target neurons of the developing cortex rather than cortical progenitors (Wu et al., 2005). However, the impact of conditional *Nex–$CB_1^{-/-}$* deletion during cortical development is unknown. Complete *$CB_1^{-/-}$* mice showed reduced progenitor proliferation in the developing cortex (Aguado et al., 2005), whereas no differences in progenitor cell proliferation were evident between *$CB_1^{f/f}$* and *Nex–$CB_1^{-/-}$* mice (Fig. 5*A*,*B*). Immunofluorescence analysis showed that Cre expression at E14.5 occurred in postmitotic areas of the developing cortex beyond the basal edge of the SVZ, as identified by the expression pattern of its marker Tbr2 (Fig. 5*C*). *In situ* hy-

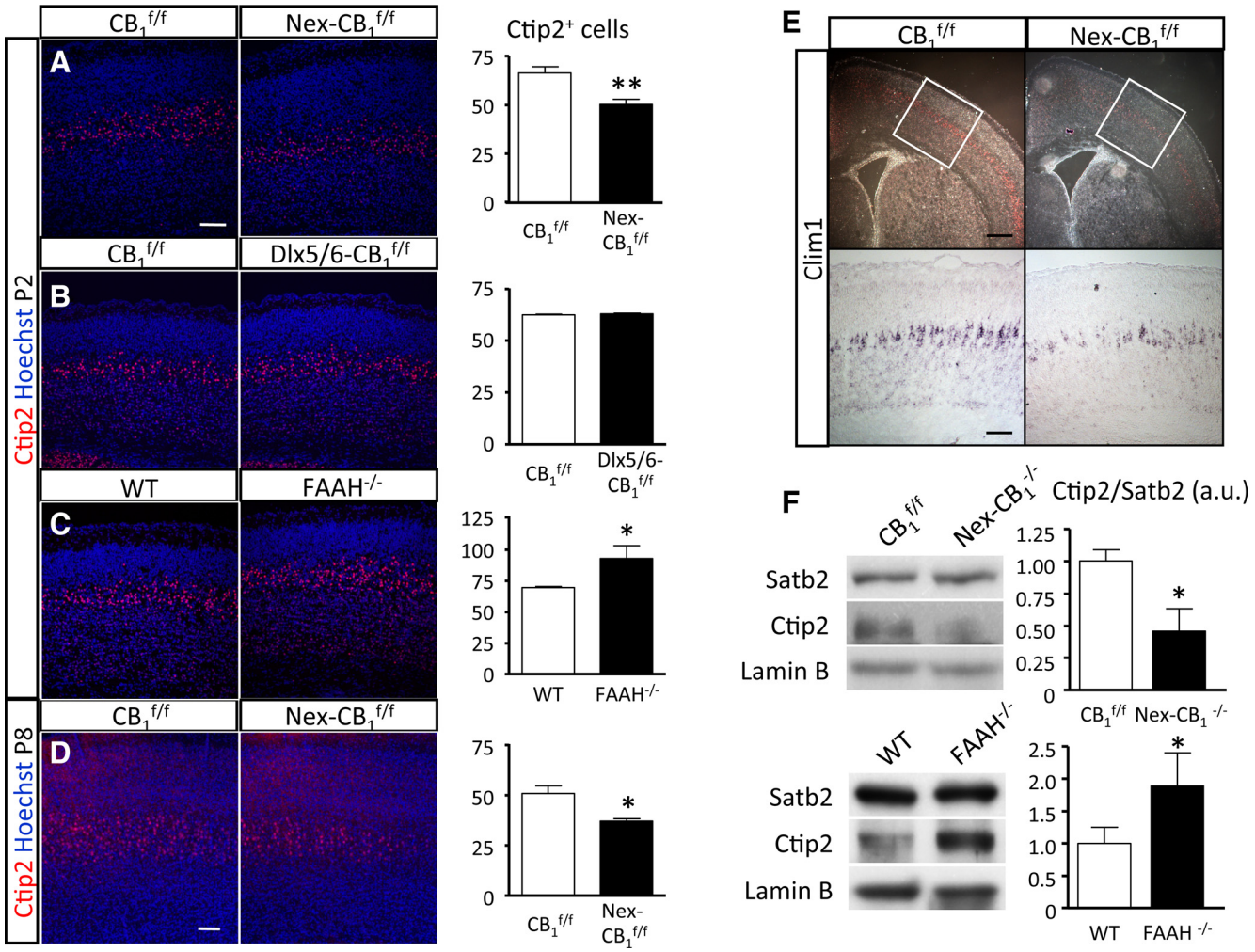

Figure 6. CB_1 receptor signaling regulates the generation of deep-layer Ctip2$^+$ neurons. ***A–D***, *Nex–$CB_1^{-/-}$*, *Dlx5/6–$CB_1^{-/-}$*, and *$FAAH^{-/-}$* mice and their respective wild-type (*$CB_1^{f/f}$* and WT) littermates were analyzed at P2, and Ctip2$^+$ neurons were quantified in 50-μm-wide cortical columns ($n = 8$ and 9, 2 and 3, and 2 and 3, respectively, for each group). *Nex–$CB_1^{-/-}$* and WT littermates were also analyzed at P8 (***D***; $n = 4$ for each group). Representative images are shown (left). ***E***, *In situ* hybridization of Clim1 at P2 in *$CB_1^{f/f}$* and *Nex–$CB_1^{-/-}$*. ***F***, Western blot analyses of nuclear extracts obtained from P2 cortices of *Nex–$CB_1^{-/-}$* and *$FAAH^{-/-}$* mice compared with their corresponding WT littermates. The relative protein levels of Ctip2 and Satb2 were quantified after densitometry, and loading control was performed with anti-lamin B1 antibody. a.u., Arbitrary units. Scale bars: ***A***, 50 μm; ***D***, 150 μm; ***E***, 150 (top) and 50 (bottom) μm.* $p < 0.05$, ** $p < 0.01$ versus WT mice sections.

bridization experiments at different stages demonstrated that *Nex–$CB_1^{-/-}$* mice preserved CB_1 expression at early proliferative stages in VZ/SVZ cells and only postmitotic neuroblasts lost their expression (Fig. 5*D*). In agreement with the notion that the CB_1 receptor regulates neuronal differentiation independently of its actions on the progenitor cell pool, the observed increase in Satb2$^+$ cells in *$CB_1^{-/-}$* was not attributable to its aberrant expression in apical or basal progenitor cells (data not shown). These findings demonstrated that the combined use of conditional *Nex–$CB_1^{-/-}$* and complete *$CB_1^{-/-}$* mice allows discriminating between CB_1 receptor actions in VZ/SVZ neural progenitor populations and CB_1-receptor-mediated regulation of differentiating postmitotic neuronal cells.

Deficient development of deep-layer Ctip2$^+$ neurons in the absence of the CB_1 cannabinoid receptor

To avoid the possible confounding interactions with impaired progenitor proliferation that occur in *$CB_1^{-/-}$* mice, we therefore turned to investigate the development of deep-layer Ctip2$^+$ neurons in *Nex–$CB_1^{-/-}$* mice. The number of Ctip2$^+$ cells at P2 along the cortical plate was reduced in *Nex–$CB_1^{-/-}$* mice compared with their WT littermates (Fig. 6*A*). Of importance, this effect was still evident at P8 (Fig. 6*D*). The direct regulatory action of CB_1 receptor in the specification of the pyramidal lineage was confirmed by analyzing *Dlx5/6–$CB_1^{-/-}$* mice, which lack CB_1 in GABAergic neurons of the forebrain (Monory et al., 2006). CB_1 receptor deletion in the GABAergic lineage did not interfere with the generation of Ctip2$^+$ cells (Fig. 6*B*). Substantiating a putative regulatory action of the eCB tone in cortical layer specification, mice deficient in fatty acid amide hydrolase (FAAH), a major eCB-degrading enzyme, showed an increased number of Ctip2$^+$ cells and, therefore, an opposite phenotype to *Nex–$CB_1^{-/-}$* mice (Fig. 6*C*). In addition, *in situ* hybridization for Clim1 (Ldb2), a transcription factor that labels subcerebral projection neurons of layer 5 (Azim et al., 2009), revealed a severe reduction of Clim1$^+$ neurons in *Nex–$CB_1^{-/-}$* mice (Fig. 6*E*). Furthermore, Western blot analysis of nuclear extracts from *Nex–$CB_1^{-/-}$* cortices showed reduced Ctip2 protein levels relative to Satb2 expression, whereas the opposite was observed in *$FAAH^{-/-}$* cortical extracts (Fig. 6*F*).

The CB_1 cannabinoid receptor regulates subcerebral projection neuron development

The alterations induced by CB_1 receptor inactivation in the progenitor layer specification program prompted us to investi-

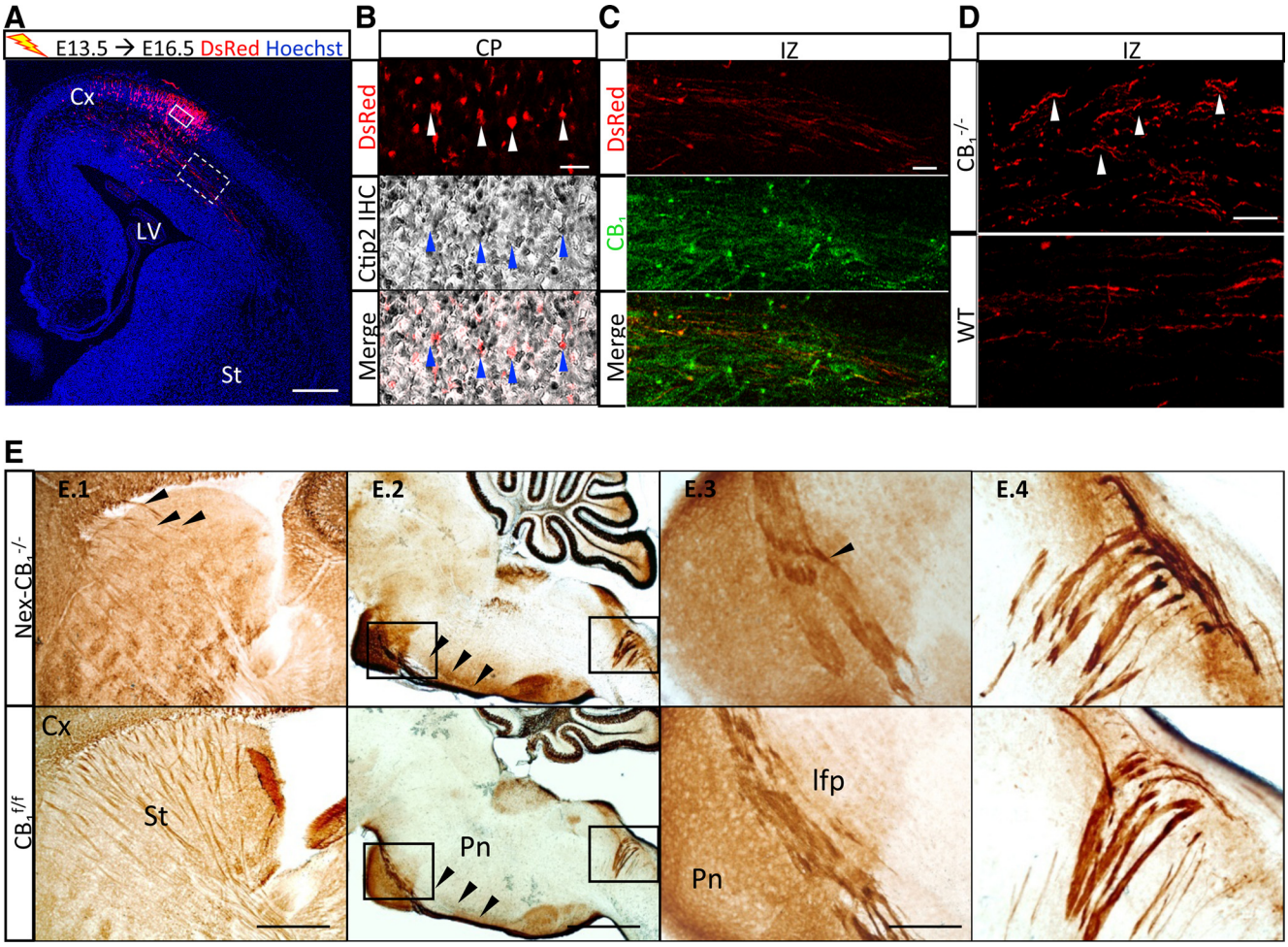

Figure 7. CB_1 receptor signaling controls axon navigation of CSMNs. $CB_1^{-/-}$ mice and WT littermates were electroporated *in utero* at E13.5 with pCAG–DsRed and fluorescence analysis was performed 3 d later. ***A***, Low-magnification image indicating the position of the analyzed insets (***B***, ***C***, solid and dashed lines, respectively). ***B***, $DsRed^+$ somata in the cortical plate show colocalization with Ctip2 immunoreactivity (middle and bottom). ***C***, Representative image of $DsRed^+$ axons expressing CB_1 receptors in the IZ of WT mice. ***D***, Navigating $DsRed^+$ axons in the IZ of WT and $CB_1^{-/-}$ mice (arrowheads). Representative images of each genotype are shown ($n = 3$). ***E***, PKCγ immunostaining images in sagittal brain sections of *Nex–*$CB_1^{-/-}$ and $CB_1^{f/f}$ mice at P8 ($n = 4$ for each group). Aberrant axonal trajectories (arrowheads) were found in corticospinal tracts in the corticostriatal junction (***E.1***). The corticospinal tract is defined in the hindbrain of *Nex–*$CB_1^{-/-}$ and control mice (***E.2***, arrowheads), but aberrant axonal fasciculation was detected in corticospinal tracts of *Nex–*$CB_1^{-/-}$ animals as they traverse the pons (***E.3***) and reach the spinal cord (***E.4***). CC, Corpus callosum; Cx, cortex; lfp, longitudinal fasciculus of the pons; LV, lateral ventricle; CP, cortical plate; Pn, pons; St, striatum. Scale bars: ***A***, ***E.3***, ***E.4***, 200 μm; ***B***, ***C***, 50 μm; ***D***, 25 μm; ***E.1***, 500 μm; ***E.2***, 1 mm.

gate its impact on axonal projection and connectivity. In line with previous observations (Mulder et al., 2008; Wu et al., 2010), aberrant corticofugal projections were found upon CB_1 deletion. Immunofluorescence microscopy analysis of the L1 neural cell adhesion molecule revealed subcortical projection deficits in P2 $CB_1^{-/-}$ and *Nex–*$CB_1^{-/-}$ mice but not in *Dlx5/6–*$CB_1^{-/-}$ mice (data not shown). The corticostriatal boundary of *Nex–*$CB_1^{-/-}$ mice showed altered axonal trajectories, and, in the intermediate zone (IZ), disorganized and enlarged fascicles were evident (data not shown). These results, together with the involvement of the CB_1 receptor in the differentiation of deep-layer $Ctip2^+$ neurons, from where corticospinal projections arise (Molyneaux et al., 2007), prompted us to investigate the role of the CB_1 receptor in subcerebral axonal projections. We performed *in utero* electroporation experiments with pCAG–DsRed in $CB_1^{-/-}$ mice at E13.5 and analyzed $DsRed^+$ projections at E16.5 (Fig. 7*A*). The corticospinal nature of DsRed-labeled axons was confirmed by the $Ctip2^+$ immunoreactivity shown by $DsRed^+$ somata (Fig. 7*B*). In WT embryos, labeled projecting axons expressed CB_1 receptor (Fig. 7*C*) and showed straight trajectories, whereas profound alterations of navigating $DsRed^+$ axons were observed at the IZ of CB_1-deficient mice (Fig. 7*D*). To confirm more precisely the exact nature of these subcortical projection alterations, we performed immunohistochemical analysis of PKCγ, because this protein is present in corticospinal tracts. Similar to the aberrant pattern of L1 immunofluorescence, abnormal axonal trajectories of PKCγ-labeled tracts in the corticostriatal junction were evident at P8 in *Nex–*$CB_1^{-/-}$ mice compared with WT littermates (Fig. 7*E.1*). Although no major alterations of the corticospinal tract in the posterior hindbrain of *Nex–*$CB_1^{-/-}$ were observed, these animals had aberrant CSMN projections as axons traverse the pons (Fig. 7*E.2*,*E.3*), those projections reaching the spinal cord in a notably less organized manner (Fig. 7*E.4*).

To unequivocally ascribe a role for the CB_1 receptor in the development of layer 5 subcerebral neurons, we took advantage of Thy1–YFP–H mice, in which the expression of the fluorescent protein under the control of the neuronal promoter of the *Thy-1* gene (encoding Thy-1 membrane glycoprotein precursor) occurs selectively in layer 5 projection neurons, thus allowing the visualization of corticospinal tracts (Feng et al., 2000; Tomassy et al.,

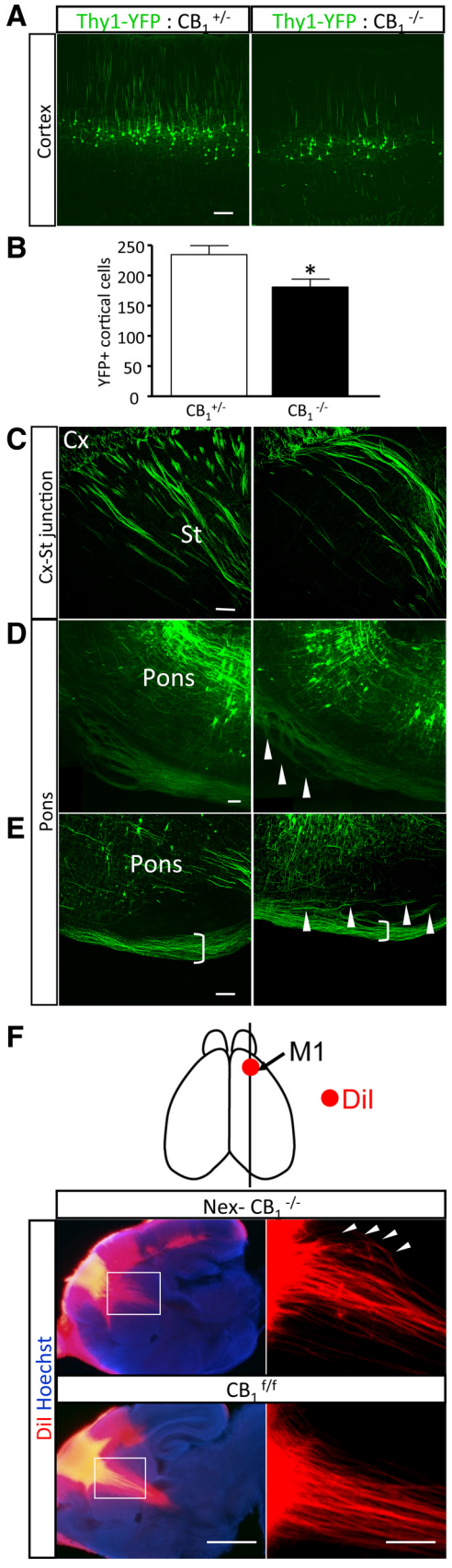

Figure 8. CB_1-deficient Thy1–YFP mice show alterations of subcerebral projection neurons. ***A***, ***B***, Confocal fluorescence images of sagittal sections of P21 cortices from *Thy1–YFP:*$CB_1^{+/-}$ and *Thy1–YFP:*$CB_1^{-/-}$ littermates. Fluorescent somata of putative CSMNs in layer 5 were quantified. $n = 2$ and 3, respectively. ***C***, ***D***, Fluorescence images illustrating corticospinal tract at the level of the corticostriatal junction and pons. ***E***, Confocal images at a more caudal level of the pons. Arrowheads indicate major alterations observed in *Thy1–YFP:*$CB_1^{-/-}$. ***F***, Projected images of DiI-labeled corticospinal tracts originating from the M1 cortex in $CB_1^{f/f}$ and *Nex–*$CB_1^{-/-}$ mice at P2. Inset shows misrouted fibers (arrowheads) found in the corticostriatal junction and traversing the striatum proximal to the cortex. Cx, Cortex; St, striatum. $^*p < 0.05$. Scale bars: ***A***, ***C***, ***E***, 150 μm; ***D***, 250 μm; ***F***, 1 mm and inset, 200 μm.

2010). We crossed $CB_1^{-/-}$ and Thy1–YFP–H mice to study the specification and development of corticospinal neurons and axonal tracts in the absence of the CB_1 receptor. At P21, a significant reduction in fluorescently labeled somata was evident in cortical layer 5 (Fig. 8*A*,*B*), whereas no changes were observed in the hippocampus (data not shown). CB_1 receptor deletion induced an aberrant phenotype of subcerebral projection neurons similar to that observed by PKC γ immunohistochemistry (Fig. 7). Thus, misrouted axons were visible while traversing the internal capsule in the corticostriatal junction in $CB_1^{-/-}$:Thy1–YFP–H mice (Fig. 8*C*), axons that reached the pons were reduced, and the remaining ones showed fasciculation alterations (Fig. 8*D*,*E*). Finally, in agreement with a role of the CB_1 receptor in subcerebral projection neuron development, anterograde DiI tracing from the motor cortex of *Nex–*$CB_1^{-/-}$ mice confirmed the existence of misrouted fibers branching off the tracts and deviating from their normal path at the corticostriatal junction (Fig. 8*F*).

The CB_1 cannabinoid receptor regulates CSMN function

The decreased Ctip2$^+$ cell population and the disruption of subcerebral axonal projections observed in $CB_1^{-/-}$ mice prompted us to investigate whether these alterations of CSMN projections result in defective cortical motor function. Evaluation of the skilled pellet-reaching task, which is dependent on CSMN-mediated connectivity (Tomassy et al., 2010), revealed that adult $CB_1^{-/-}$ mice had a remarkable impairment in fine motor function (Fig. 9*A*). The total number of trials performed during the skilled task was not significantly different between the two groups of mice, and unskilled motor activity was not affected either (Fig. 9*C*,*D*), thus indicating the selectivity of the skilled motor function deficits. Additional support for this selectivity was provided by the observation that the general motor activity, including total distance traveled, resting time, and fast movements (ActiTrack test), as well as motor coordination (RotaRod test), did not differ between $CB_1^{-/-}$ and WT mice (Fig. 10*A*). Moreover, and in concert with the neuroanatomical findings described above, the deficits in skilled motor activity observed in $CB_1^{-/-}$ mice were recapitulated in *Nex–*$CB_1^{-/-}$ mice (Fig. 9*B*).

Additional validation of the abnormal skilled motor function phenotype found in $CB_1^{-/-}$ animals was obtained by using the staircase test, which also reflects fine motor activity and is useful to evaluate motor impairment after cortical lesions (Brooks and Dunnett, 2009). $CB_1^{-/-}$ and *Nex–*$CB_1^{-/-}$ mice had a lower performance than their respective WT littermates in their ability to grasp the most difficult food pellets (steps 4–8) (Fig. 9*E*–*G*). Moreover, we observed that retrieval of non-challenging pellets in the staircase test (steps 1–3) was not different among genotypes (Fig. 9*H*), thus confirming the selective impairment of fine motor activity. Finally, the patch-removal task, which also evaluates sensorimotor function, was also used. *Nex–*$CB_1^{-/-}$ mice were significantly less efficient than their WT littermates in removing a piece of adhesive tape from their hindpaws, as demonstrated by the higher number of contacts required for patch removal (Fig. 10*B*, left). This decreased performance of *Nex–*$CB_1^{-/-}$ mice in patch removal reflected a fine motor function impairment rather

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than an altered perception of the patch, as indicated by the quantification of the latency time for the first attempt to remove the patch and the total time spent removing the patch, which were not significantly different between both genotypes (Fig. 10*B*, middle and right).

Discussion

Recent studies have shown that the CB_1 receptor regulates neural progenitor proliferation and axonal navigation, thus suggesting an instructive role of the eCB system in nervous system development (Harkany et al., 2007). The present study provides new insights into the developmental role of the CB_1 receptor, revealing that it exerts a pivotal role in regulating the proneurogenic transcription factor code that controls cortical neuron differentiation. In particular, we show here that the CB_1 receptor (1) tunes the differentiation balance of deep- and upper-layer cortical projection neurons, (2) is coupled to the regulation of the Ctip2–Satb2 transcriptional regulatory code, (3) plays a regulatory role in CSMNs development, and, as a consequence, (4) is required for the correct function of the mature CNS. Thus, cortical development is regulated by CB_1 receptor signaling through a dual progenitor-dependent and progenitor-independent mechanism of action. Although the CB_1 receptor present in neural progenitors promotes cortical progenitor self-renewal and VZ/SVZ proliferation in a cell-autonomous manner (Aguado et al., 2005; Mulder et al., 2008), the CB_1 receptor expressed in radially migrating neuroblasts controls the intrinsic neurogenic transcriptional program involved in the appropriate balance of cortical layer differentiation (present study) by sensing the extracellular eCB neurogenic niche.

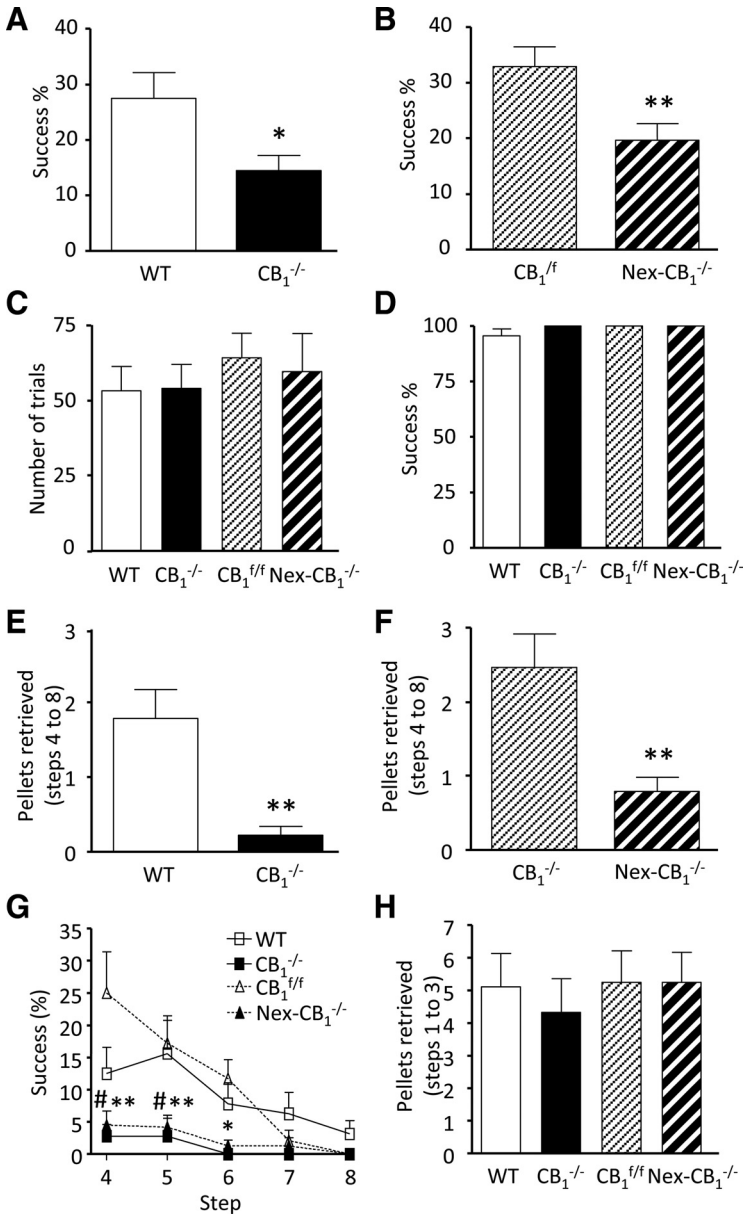

Figure 9. CB_1-deficient mice show impaired corticospinal motor function. ***A–C***, $CB_1^{-/-}$, *Nex–*$CB_1^{-/-}$, and their corresponding wild-type littermates (WT and $CB_1^{f/f}$, plain and stripped, black and white bars, respectively) were analyzed in the skilled pellet-reaching task test. Fine motor skill was evaluated in the animal groups after cage habituation and training. The percentage of pellets retrieved (***A***, ***B***) and the total number of trials performed during the skilled task (***C***) are shown. ***D***, Unskilled motor function was assessed, and the percentage of pellets retrieved was calculated. ***E***, ***F***, Mice were subjected to the staircase pellet-reaching test, and the sum of pellets retrieved from challenging steps (from 4–8) was compared between $CB_1^{-/-}$ or *Nex–*$CB_1^{-/-}$ mice and their control littermates. ***G***, The percentage of success for each step among the different genotypes was quantified. ***H***, The number of pellets reached in non-challenging steps 1–3 did not differ between groups. $n = 9$ ($CB_1^{-/-}$ and WT) and $n = 17$ and 16 (*Nex–*$CB_1^{-/-}$ and $CB_1^{f/f}$) for each group. $^*p < 0.05$, $^{**}p < 0.01$ versus WT, $^{\#}p < 0.05$ versus $CB_1^{f/f}$ littermates.

CB_1 cannabinoid receptor-dependent regulation of the neurogenic niche during neocortical development

During early cortical development, the CB_1 receptor is not expected to exert the classical neuromodulatory role of the eCB system in the mature adult brain, because synaptic maturation and activity is not yet completed. Therefore, during corticogenesis, CB_1 receptor-mediated neural fate decisions occur in a cell-autonomous manner (Aguado et al., 2005, 2007). Later, CB_1 receptor regulation of synaptic plasticity may turn a predominant mechanism of action, as exemplified at postnatal stages when the CB_1 receptor regulates the whisker map development of the somatosensory cortex in a neuronal activity-dependent manner (Li et al., 2009).

Here, we demonstrate that the CB_1 receptor allows differentiating neurons to transduce information from the surrounding neurogenic niche by modulating the intrinsic fate determinants involved in the developmental gene expression program of neuronal differentiation. It remains to be determined which signals control the production of eCBs released by neural progenitors and differentiating neurons (Aguado et al., 2005). Cortical progenitors express the enzyme involved in the synthesis of 2-arachidonoylglycerol (2AG) diacylglycerol lipase (Berghuis et al., 2007), which is downregulated during progenitor cell differentiation (Walker et al., 2010). In addition, the expression of the degradation enzymes FAAH and monoacylglycerol lipase (MAGL) during embryonic neuronal differentiation allows the delicate control of eCB levels (Aguado et al., 2005; Mulder et al.,

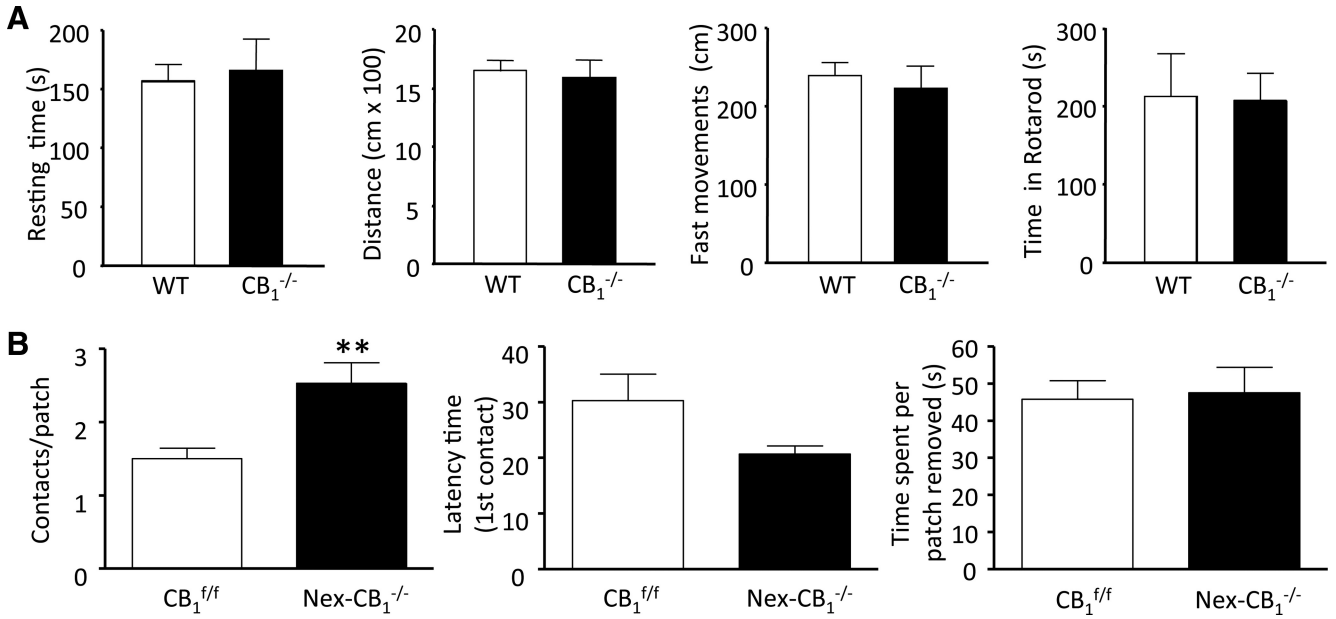

Figure 10. Behavioral characterization of $CB_1^{-/-}$ and $Nex\text{–}CB_1^{-/-}$ mice. ***A***, General motor behavior in $CB_1^{-/-}$ mice and WT littermates. Adult mice were analyzed for ambulation in the open-field test and for motor coordination in the RotaRod test. $n = 9$ for each group. ***B***, Patch-removal analysis confirms the deficient fine motor function of $Nex\text{–}CB_1^{-/-}$ mice compared with their $CB_1^{f/f}$ littermates. The number of contacts required for patch removal, the latency time for the first patch contact, and the time spent per patch were quantified in each genotype. $n = 16$ and 17 for each group. $^{**}p < 0.01$ versus $CB1^{f/f}$ mice.

2008; Keimpema et al., 2010). Regulation of 2AG availability by MAGL is responsible for the regulation of axonal growth by CB_1 receptor signaling (Keimpema et al., 2010). 2AG produced in thalamocortical axons can activate the CB_1 receptor present on growth cones of corticothalamic tracts, thus contributing to their correct integration and coordination (Wu et al., 2010). In addition, eCB depletion by the overexpression of the FAAH enzyme inhibits radial migration, suggesting a role of the endogenous cannabinoid tone in pyramidal neuron migration (Mulder et al., 2008). Overall, these evidences indicate that regulated expression and activity of the different signaling elements of the eCB system are part of the differentiation program of projection neurons.

CB_1 cannabinoid receptor-dependent regulation of forebrain neurogenic transcription network drives CSMN differentiation

Analyses of CB_1-deficient mice revealed that neuronal differentiation is altered, as shown by the delayed distribution of postmitotic $Tbr1^+$ neuroblasts and fate decision changes mediated by the Ctip2/Satb2 code. Nex–Cre-driven deletion of CB_1 allowed us to dissect the role of CB_1 solely in neuronal differentiation because, in $Nex\text{–}CB_1^{-/-}$ mice, cortical progenitors preserve CB_1 receptor expression and cell proliferation is not affected. The prominent role of the gene expression program regulated by the Ctip2 pathway in subcerebral CSMN generation (Arlotta et al., 2005; Chen et al., 2008; Tomassy et al., 2010) and the ability of the CB_1 receptor to control this transcriptional regulation axis reveal that the CB_1 receptor constitutes a novel signaling platform involved in the appropriate corticofugal neuronal development and skilled motor function by tuning subcerebral-projecting versus callosal neuron-projecting differentiation.

In $CB_1^{-/-}$ mice, the transcriptional repressor Satb2 is deregulated and $Satb2^+$ cells are expanded and occupy developing deep layers, in which they are normally absent (Alcamo et al., 2008; Britanova et al., 2008). Because of the inhibitory effect of Satb2 on Ctip2 expression (Alcamo et al., 2008; Britanova et al., 2008), CB_1 receptor deletion led to a reduction of $Ctip2^+$ cells generated in layer 5. These results suggest the existence of complementary changes in transcriptional regulators of CSMN specification during CB_1 receptor loss. In CB_1-deficient mice, reduced Ctip2 and Fezf2 expression was observed. Fezf2 acts upstream of Ctip2 and is necessary and sufficient to induce subcortical axonal projection neurons of deep cortical layers (Molyneaux et al., 2005; Chen et al., 2008). Upper-layer neurons develop normally with concomitant Fezf2 downregulation, whereas its overexpression induces the excessive generation of corticospinal projections from where callosal projections normally arise (Molyneaux et al., 2005). Of importance, Tbr1 and Fezf2 mutually repress each other, and this regulatory mechanism is essential for the development of the corticospinal tract (McKenna et al., 2011). Thus, alterations of the Tbr1–Fezf2 transcriptional balance may also contribute to the deficits in CSMN specification observed in the absence of CB_1 receptor signaling. In addition, loss of CB_1 receptor also affected Clim1, another member of the subcerebral specification transcriptional regulation program that allows distinction between callosal and subcerebral layer 5 projection neurons (Azim et al., 2009). Of note, our findings of unbalanced transcriptional regulation of projection neuron differentiation *in vivo* are supported by *in vitro* studies of CB_1 receptor-induced regulation of transcriptional activity and neuronal differentiation. Considering our previous data supporting a role of the eCB system in radial migration during corticogenesis (Mulder et al., 2008) and its ability to regulate downstream signaling systems that coordinate cell migration and neural cell differentiation, such as the mammalian target of rapamycin signaling pathway (Puighermanal et al., 2009; Palazuelos et al., 2012), a plausible hypothesis to be tested in the future would be the potential relationship between CB_1 receptor regulation of radial migration and neuronal differentiation.

Role of the CB_1 cannabinoid receptor in CSMN development and function: pathophysiological implications

Elucidating the contribution of CB_1 receptor expressed during brain development to the function of the future adult brain is a challenging task with important biomedical implications. In the present study, we provide evidence for the existence of functional impairments in the brains of adult CB_1 receptor-deficient mice as a consequence of alterations in developmental neuronal differentiation. Adult $CB_1^{-/-}$ mice show defective skilled motor function that ensues alterations of corticospinal tract development associated with the regulatory role of the CB_1 receptor of Ctip2/Satb2 transcriptional activity. Skilled motor behavior relies on the establishment of appropriate corticospinal connectivity, and even relatively mild alterations of the corticospinal tract can critically impair fine motor skills (Tomassy et al., 2010).

In addition to the skilled motor phenotype described herein, an emerging scenario suggests that genetic alterations or polymorphisms of CB_1 and eCB-synthesizing/degrading enzymes can contribute to changes, often subtle, in forebrain development, which might in turn contribute to the susceptibility to a variety of psychiatric disorders in the adult brain (Galve-Roperh et al., 2009; Fiskerstrand et al., 2010). Likewise, changes in prenatal CB_1 receptor function may conceivably ensue during pregnancy during mother's consumption of cannabinoid receptor agonists or antagonists (Jutras-Aswad et al., 2009) or exposure to other xenobiotics that interact with the eCB system, such as ethanol or organophosphorous pesticides (Nomura et al., 2008). Human and animal model studies have shown that loss of function of neuronal specification determinants (e.g., Tbr1, Satb2, Ctip2) results in alterations of cortical development and neurogenesis, which produces in the adult brain severe consequences in behavioral processes, such as motor control, cognition, epileptogenesis, and sensorimotor integration (Tomassy et al., 2010; Saito et al., 2011). Deregulated gene expression levels of deep-neuronal specification factors are associated with temporal lobe epilepsy and a particular form of autism (Pasca et al., 2011; Rossini et al., 2011).

Defective genesis and maturation of glutamatergic projection neurons alter the excitation/inhibition neurochemical balance and influence the interneuron populations of the cortical plate (Sessa et al., 2010; Lodato et al., 2011), and these alterations could contribute to the etiology of a large variety of disorders, such as epilepsy and schizophrenia (Lewis and Sweet, 2009). The participation of the CB_1 receptor in deep-layer neuronal differentiation and function reported here may provide a better understanding of the potential involvement of the eCB system in the neurodevelopmentally evoked susceptibility to motor neurodegenerative disorders (Blazquez et al., 2011), seizure occurrence (Marsicano et al., 2003), and psychiatric disorders (Jutras-Aswad et al., 2009).

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Impaired corticospinal motor neuron development and function and increased seizure susceptibility in prenatally Δ^9-tetrahydrocannabinol-exposed mice

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The endocannabinoid system plays a key neuromodulatory role in the mature brain. Besides, it is present and functional since early stages of prenatal development, and it has been shown to exert a key regulatory role in several processes taking place, specially, in CNS development. CB_1 cannabinoid receptors are targeted both by endogenous ligands and by Δ^9-tetrahydrocannabinol (THC), the active constituent of marijuana. Prenatal exposure to THC has been shown to predispose the developing CNS to a pathological configuration, likely by blurring CB_1-dependent processes in cortical development. In this study, we analyzed the impact that THC embryonic exposure might have on the generation and specification of corticospinal motor neurons (CSMNs), a process previously known to be modulated by CB_1 signaling. Therefore, a THC exposure protocol aimed at affecting preferentially CSMN generation and early specification steps was followed. We found a significantly reduced number of CSMNs upon THC gestational exposure, and detected alterations in their axonal projections. As a consequence, fine motor control, critically dependent on CSMN function, was impaired in THC-administered offspring. Interestingly, CNS developmental traits upon THC exposure resulted in an increased susceptibility to PTZ-induced seizures. Our data suggest that THC affects cortical development by acting as a functional antagonist at CB_1 receptors, impeding the physiologically temporal- and spatially confined CB_1 function.

INTRODUCTION

The mammalian cerebral cortex comprises 6 layers, populated by different excitatory pyramidal neuron subtypes with particular birthdate, molecular determinants and connectivity pattern. Each pyramidal neuron lineage is born in a temporally-restricted manner, and populate the cortex following an inside-out pattern. First to arrive, corticothalamic and subcerebral projection neurons, occupy deep layers LVI and LV, respectively. Locally projecting stellate cells are restricted to LIV and associative and callosal projection neurons are preferentially located in upper layers LII/III (Greig, Woodworth, Galazo, Padmanabhan, & Macklis, 2013). Extracellular cues like Notch ligands, reelin, and neurotrophins contribute to the coordination of the choreographed population diversity of the developing cerebral cortex, therefore refining the hodological, laminar and molecular features of each neuronal subtype. Alterations of several processes taking place during cortical development are known to be the cause of an increasing number of neuropathologies, including neurodegenerative motor disorders, such as certain forms of motor neuron disorders such as primary lateral sclerosis affecting upper motor neurons (Ozdinler et al., 2011; Robberecht & Philips, 2013), and epilepsy (Roberts, Royston, & Gotz, 1995; Sisodiya, 2004), among many others.

The endocannabinoids, acting through their CB_1 receptors, act as key neuromodulators at mature synapses (Castillo, Younts, Chavez, & Hashimotodani, 2012; Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). CB_1 receptors are also present and functional from early developmental stages (Galve-Roperh et al., 2013), and play an important role in the regulation of several key processes taking place during cerebral cortex development including: i) progenitor cell self-renewal and neurogenesis (Aguado et al., 2005; Diaz-Alonso et al., 2014), ii) neuronal migration (Berghuis et al., 2005; Mulder et al., 2008), (Díaz-Alonso et al. unpublished results), and iii) neuronal and glial specification (Aguado et al., 2006; Diaz-Alonso et al., 2012b). A crucial role has been assigned to the CB_1

receptor in the control of corticofugal projection neuron development. Therefore CB_1 receptor signaling is required for their proper axonal navigation and fasciculation (Mulder et al., 2008; Wu et al., 2010). Among the corticofugal pyramidal neuron sublineages, CB_1 function has been shown to be essential for subcerebral projection neuron development (Diaz-Alonso et al., 2012b). CB_1-deficiency affects corticospinal motor neuron (CSMN) specification at the molecular level, turning the transcription factor network governing pyramidal neuron identity towards Satb2-driven callosal identity, instead of Ctip2-driven corticospinal, projection neuron specification. This molecular switch results in decreased number of CSMNs, alterations of corticospinal axonal projections and, as a consequence, the impairment of the fine motor control that critically dependens on CSMN function.

The CB_1 cannabinoid receptor is targeted also by **Δ^9-THC**, the main psychoactive constituent of marijuana. Cannabis is, by far, the most commonly used illicit drug in the West countries during pregnancy, and therefore its use during pregnancy constitute a considerable public health issue. A bulk of longitudinal human studies, as well as basic research using animal models (Jutras-Aswad, DiNieri, Harkany, & Hurd, 2009; Schneider, 2009), encouraged by the need for a better understanding of the possible impact of cannabinoid exposure on the neuropsychiatric health of the offspring, have emerged in the past decades. Many studies conclude that cannabinoid exposure during development sensitizes the CNS network to cognitive impairments (Huizink & Mulder, 2006), increases the probability of the onset of neuropsychiatric disorders, such as schizophrenia and anxiety (Jutras-Aswad et al., 2009) and may predispose to drug abuse, strikingly even in the subsequent generation (Szutorisz et al., 2014) Presumably, modifications of the endogenous cannabinoid signaling elicited by developmental exposure to cannabis, either by overactivation of cannabinoid receptors physiologically tempered or by causing desensitization and loss of function of normally active CB_1 receptors underline the molecular, anatomical and behavioral consequences of embryonic cannabis exposure, but the precise mechanisms involved remain to be completely understood (Keimpema, Mackie, & Harkany, 2011). In this study we aimed at modeling prenatal cannabinoid exposure in mice. We designed a THC administration protocol from gestational day (g.d.) 12 to g.d. 16, in order to preferentially target subcerebral projection neurons development (Greig et al., 2013). By molecular, cellular and behavioral approaches, we tested whether THC exposure in this time window affected the neurodevelopmental processes previously shown to be controlled by CB_1 receptor signaling (Diaz-Alonso et al., 2012b; Mulder et al., 2008). Interestingly, we found a substantial decrease in CB_1 levels in the cerebral cortex, thus suggesting that THC exposure causes CB_1 receptors downregulation. In agreement, cannabinoid administration mimicked the consequences of genetic CB_1 receptor loss of function in CSMN development, and caused the impairment in fine motor control in the adulthood. Moreover, the altered wiring of the cerebral cortex elicited by endogenous cannabinoid signaling alterations caused by THC exposure resulted in a sensitization to epileptic seizures in the offspring.

METHODS

Animals. Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with the European Commission regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Mice were maintained in standard conditions, keeping littermates grouped in breeding cages, at a constant temperature (20±2ºC) on a 12-h light/dark cycle with food and water *ad libitum*. The generation and genotyping of $CB_1^{-/-}$, and their respective wild-type (WT) littermate controls, has been reported elsewhere and was performed accordingly (Monory et al., 2006). Mouse embryonic tissues were obtained upon timed mating as assessed by vaginal plug observation (E0.5). THC (THC Pharm) was diluted in 0.9% NaCl (saline) solution containing 10% DMSO and 10% tween, and administered intraperitoneally to a final dose of 3mg/Kg to pregnant females 5 consecutive days, from g.d. 12 to g.d. 16 (Fig. 1A). Control mice were injected with vehicle solution.

Immunofluorescence and confocal microscopy. Coronal brain slices (30 µm-thick) were processed as previously described (Diaz-Alonso et al., 2012a). Cortical layers were identified by their discrete cell densities as visualized by DAPI counterstaining. Immunofluorescence was performed, after blockade with 5% goat serum, by overnight incubation at 4ºC with primary antibodies against CB_1 (Frontier Institute, Japan), ER81 (Abcam) and Neurofilament 2F11 (Dako), followed by incubation for 1 h at room temperature with secondary antibodies. The appropriate anti-mouse, guinea pig and rabbit highly cross-adsorbed AlexaFluor secondary antibodies (Invitrogen, Carlsbad, CA) were used. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with 2 passes by Kalman filter and a

1024X1024 collection box. Immunofluorescence of cortical sections was performed along the rostral to caudal axis and the quantifications were carried out in equivalent sections from the mediolateral area of the motor/somatosensory cortex. Quantification of CB_1 immunorreactivity in vehicle- and THC- exposed postnatal cortex using images obtained with exactly same acquisition settings was performed with Image J software.

Immunoblot assays. Embryonic brain tissue was collected the day after last THC injection. Protein samples were prepared in lysis buffer supplemented with protease inhibitors. Equal amounts of protein samples were electrophoretically separated and transferred to PVDF membranes. After blocking, membranes were incubated ON at 4ºC with anti-CB_1 (1:1000) (Frontier Science, Japan), and anti-beta-actin (1:5000) (Cell Signaling) primary antibodies, and the corresponding secondary antibodies coupled to horseradish peroxidase. The optical density of the relevant immunoreactive bands was quantified with the gel quantification plugin of Image J software. The values for CB_1 were normalized to beta-actin detection in the same samples.

CSMN Retrograde labelling. For retrograde labeling, deeply anaesthetized mice were injected with 0,5 µl of red fluorescent microspheres (Lumafluor Inc.) into the dorsal funiculus of cervical spinal cord at P10 and perfused at P15. Brains were sectioned coronally at 30 µm, and CSMN in sensorimotor and in lateral sensory cortex were counted on every sixth section, across the entire rostrocaudal extent of the cortex, and refered to a 1mm-wide coronal cortical section.

Behavioral tests. THC of Vehicle-exposed WT and $CB_1^{-/-}$ littermates at 8 weeks of age were trained and tested for skilled reaching and staircase tests as previously described (Diaz-Alonso et al., 2012b). All tests were video recorded for subsequent analysis and double-blind quantification. Results shown correspond to the average of two tests. Additional characterization of general motor activity and exploration was performed with an ActiTrack (Panlab) device and as described previously (Blazquez et al., 2011).

Seizure induction with pentylenetetrazol. We dissolved pentylenetetrazol (PTZ, Sigma) in 0.9% saline and administered it intraperitoneally to mice at P60 at a concentration of 30 mg/kg. There was no statistically significant difference in weight or sex ratio between groups of mice. Mice were placed in Plexiglass cages, observed by an experimenter blinded to their treatment and genotype and their behavior was scored with the Racine's scale. We administered PTZ every 10 minutes until generalized seizures occurred.

Data analyses and statistics

Results shown represent the means ± S.E.M., and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way ANOVA, as appropriate. A *post-hoc* analysis was made by the Student-Neuman-Keuls test.

RESULTS

Embryonic THC administration interferes with subcerebral projection neuron development

CB_1 receptors contribute to the generation and specification of CSMNs (Diaz-Alonso et al., 2012b). In this study, we explored the potential impact of THC exposure-driven CB_1 signaling alterations on the development of this pyramidal neuron subtype. To do so, avoiding the confounding influence of cannabinoid exposure during the initial gestational stages (Galve-Roperh et al., 2013; Psychoyos, Hungund, Cooper, & Finnell, 2008), we designed a scheduled THC administration protocol consistent in the IP injection of THC –or its vehicle-, to pregnant CD1 mice during 5 consecutive days (Fig.1A), coinciding with the peak of generation of corticospinal motor neurons and the initial steps of their postmitotic specification (Molyneaux, Arlotta, Menezes, & Macklis, 2007). We employed a THC dose of 3 mg/Kg, capable for inducing the classic physiological and behavioral outcomes of THC intoxication (Little, Compton, Johnson, Melvin, & Martin, 1988) but low enough to minimize THC effects beyond CB_1 receptors. Taking into account that about one third of the THC blood content undergoes cross-placental transfer (Hurd et al., 2005), we estimate an approximate effective concentration of 1mg/Kg readily reaching the developing fetus CNS. We first quantified the generation of subcerebral projection neurons in THC- and vehicle-treated offspring by immunofluorescent detection of ER81, a *bona-fide* marker of corticofugal projection neurons somata in the cerebral cortex (Molnar & Cheung, 2006; Rouaux & Arlotta, 2013). The number of $ER81^+$ neurons was decreased THC-exposed animals when compared to their vehicle-treated controls (Fig. 1B-D). We then wanted to investigate the impact of THC administration on corticospinal motor axon projections. Therefore, we performed fluorescent-retrograde labeling from the cervical spinal cord, a commonly used method to unequivocally assess the identity of CSMNs (Arlotta et al., 2005; Ozdinler et al., 2011) to THC-exposed and vehicle treated mice. We found a significant reduction in the number of labeled

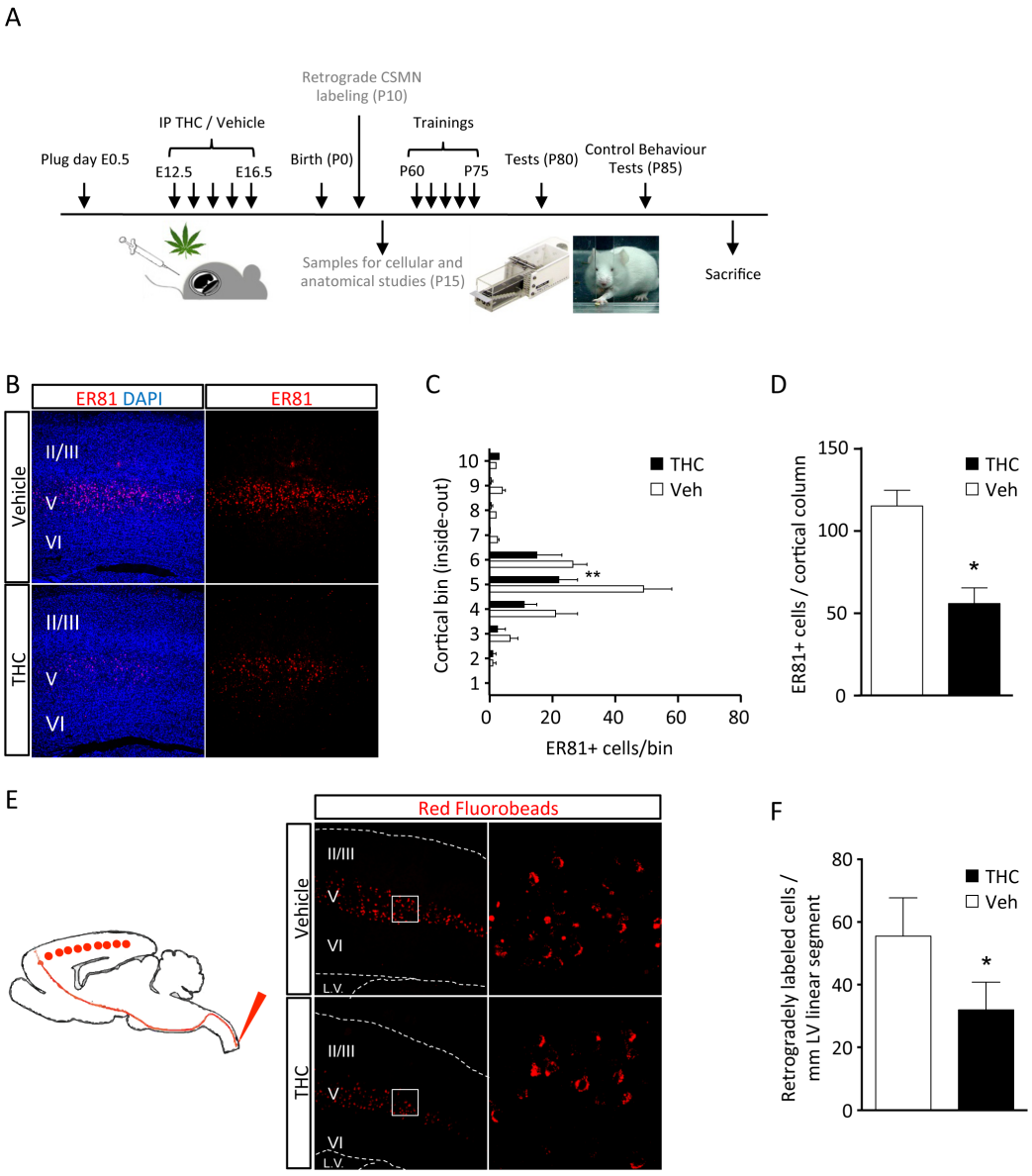

Figure 1. Embryonic THC exposure impairs CSMN development. (A), Scheme of the protocol followed for THC administration during prenatal development. (B-D), Subcerebral projection neuron somata were stained with an anti-ER81 antibody in embryonically THC- and vehicle-exposed mice at P15. Quantification was performed in 10 equally-sized bins (B). Also total numbers are given (C). (E, F), CSMN retrograde labeling was performed by injecting red fluorescent beads in the cervical spinal cord at P10, and quantifications of the number of labeled somata was performed at P15. n= 2 and 4-5 mice per group (B-D and E-F, respectively). *, $p<0.05$; **, $p<0.01$.

corticospinal somata in THC-treated mice compared with their controls (Fig. 1E-F). These results confirmed the impairment of CSMN development induced by subchronic THC embryonic administration, and indicate that reduced CSMN development results in subcerebral connectivity alterations.

Embryonic cannabinoid exposure induces skilled motor control impairment

We next examined whether CSMN function was affected upon cannabinoid gestational exposure. To assess CSMN function, we employed the skilled reaching test, a well-established behavioral test that allows the dissection of CSMN-dependent motor function reflected as the ability to retrieve a pellet of palatable food with a forelimb through a narrow slit (Tomassy et al., 2010). This behavioral procedure, as many others, can bring together a certain degree of anxiety responses, which could be potentiated by the worsening of maternal care derived from THC administration (Golub, Sassenrath, & Chapman, 1981). To avoid the possible misinterpretations derived from THC-exposure effects in mothers, we used $CB_1^{-/-}$ females, devoid of the behavioral impact of THC, crossed with $CB_1^{+/-}$ males. Therefore, we analyzed the skilled motor function in $CB_1^{+/-}$ and $CB_1^{-/-}$ offspring. As

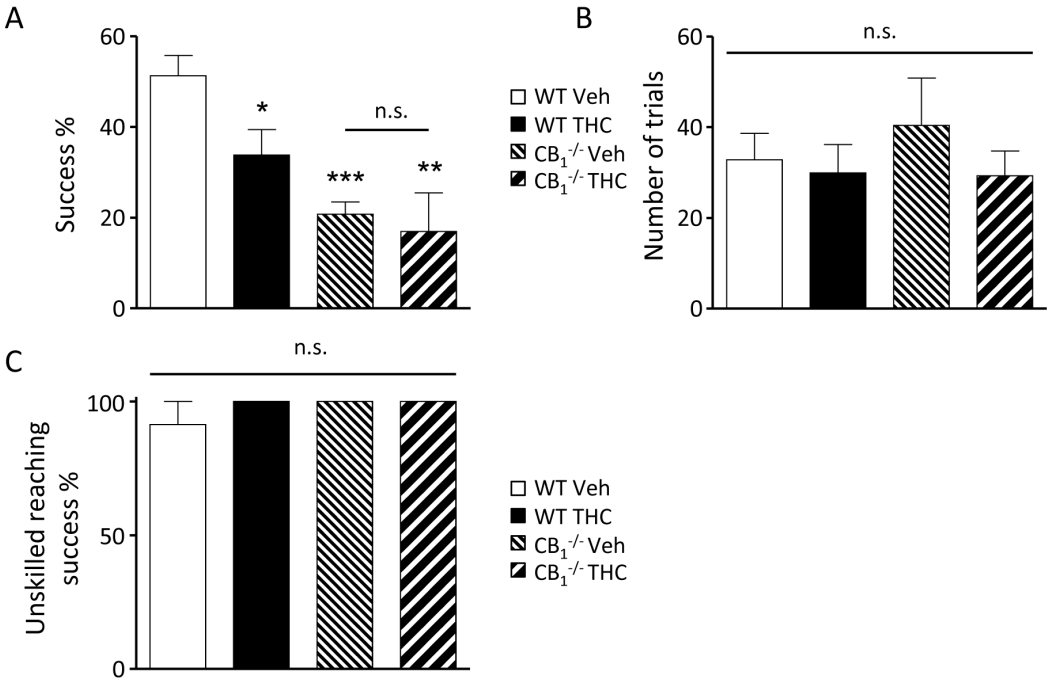

Figure 2. Skilled motor function is impaired in THC-exposed mice. (A), WT and $CB_1^{-/-}$ mice, embryonically administered THC or vehicle were tested in the skilled reaching task, after training them during 3 weeks. Their success retrieving the palatable pellets is indicated as a percentage. (B, C), Total number of trials per test (B) and success in a non-skilled task (C) were measured as control. n= 6-13 mice per group. *, p<0.05; **, p<0.01, ***, p<0,001.

shown in Fig. 2A, THC-exposed animals suffer a significant impairment in the skilled motor function in comparison to vehicle-treated mice. Noteworthy, THC exposure did not worsen skilled motor performance in CB_1-deficient mice, whereas the previously reported impairment in full and glutamatergic-specific CB_1 knockouts (Diaz-Alonso et al., 2012b) was reproduced. Importantly, neither the number of trials, nor the success in unskilled conditions (i.e., the ability to retrieve a pellet at a tongue-reaching distance) were changed among groups (Fig. 2B, C), ruling out the involvement of motivational or generalized unspecific motor alterations in the impaired skilled motor performance. In addition, we employed the staircase test, that also allows the assessment of impairments in corticospinal function. Again, a decreased performance in the staircase test was evident in THC-exposed WT mice when compared to their vehicle-treated counterparts (Fig. 3A). We confirmed previous findings of staircase test deficits in CB_1 deficient mice and, although THC treatment showed a tendency to worsen their ability to reach the pellets, differences were not statistically significant with vehicle-treated $CB_1^{-/-}$ mice. Control quantifications of unskilled reaching did not show significant differences among groups (Fig. 3B).

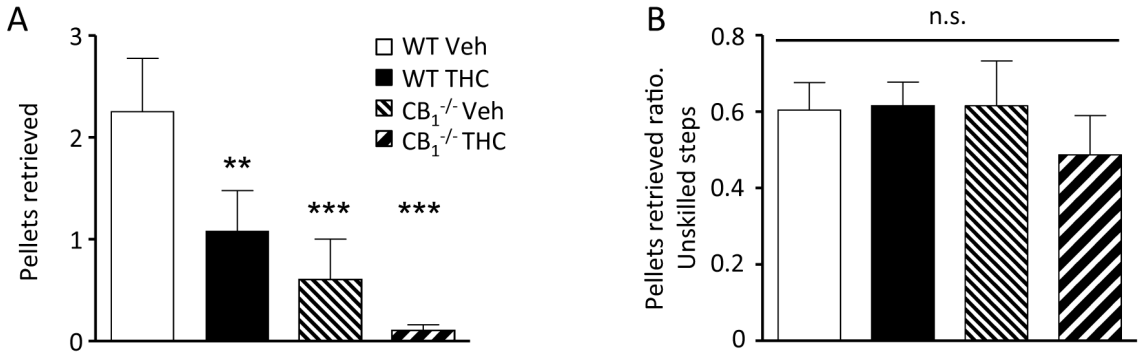

Figure 3. THC-administration impairs Staircase test performance. (A), WT and $CB_1^{-/-}$ mice, embryonically administered THC or vehicle were tested in the Staircase apparatus, after training them during 3 weeks. The number of pellets retrieved in the highly demanding steps is quantified. (B), Control quantification of the number of pellets retrieved in the non-skilled steps is also indicated. n= 6-13 mice per group. **, p<0.01, ***, p<0,001.

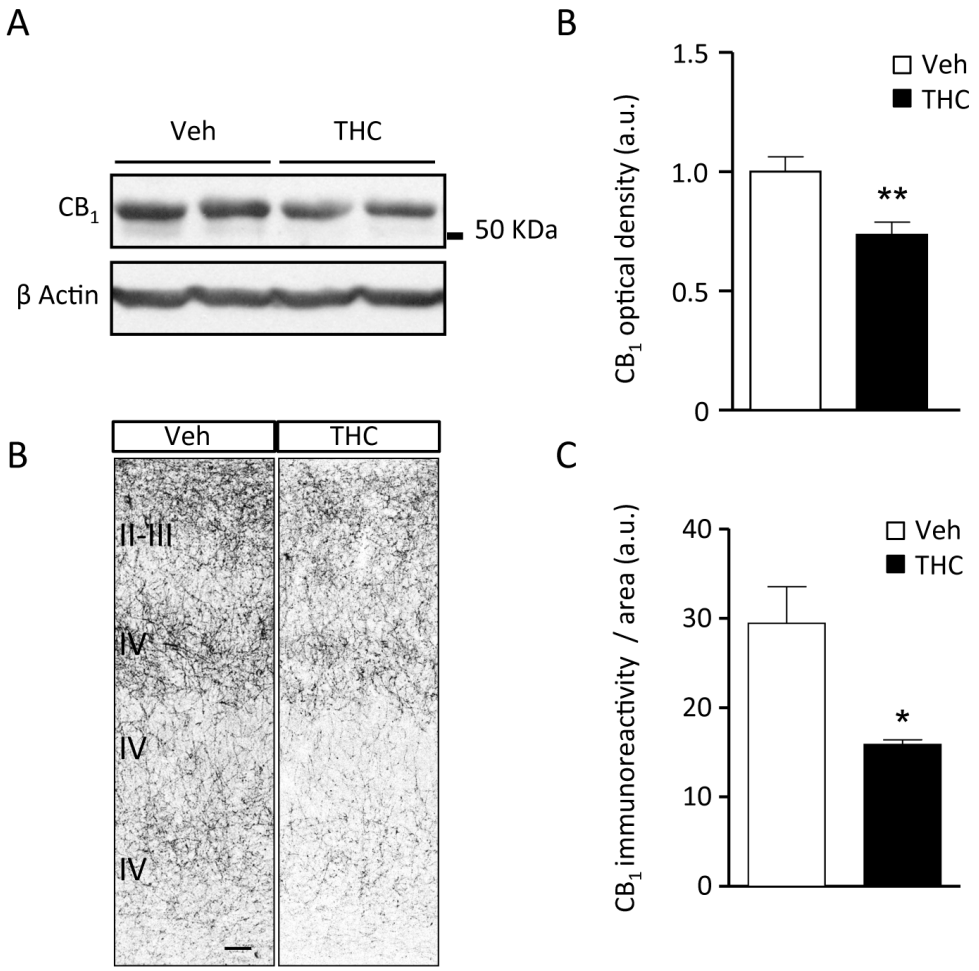

Figure 4. THC exposure during embryonic development downregulates CB_1 receptors. (A, B), CB_1 receptor expression levels were determined by western blot in E17.5 brain samples, 24h after the last THC or vehicle injection. The bands corresponding to CB_1 were quantified with Image J software and the ratio with beta-actin was calculated and compared between treatments. (C, D), CB_1 expression was assessed by immunofluorescence in the cerebral cortex of P15 mice, subjected to embryonic THC or vehicle administration. Immunoreactive area was calculated using Image J software, and referred to total area in equivalent cortical areas. n= 5-7 embryos or mice per group. Scale bar, 50mm. *,p<0,05; **,p<0,01.

THC embryonic exposure downregulates CB_1 receptors in the developing cortex

Δ^9-THC acts as a partial agonist at CB_1 receptors, with important pharmacokinetic and pharmacodynamic differences when compared with the endogenous cannabinoids. Chronic THC treatment has been shown to trigger tolerance mechanisms, manifested in a reduced response to cannabinoid administration-induced behavioral effects (Hutcheson et al., 1998). This tolerance relies, at least in part, in CB_1 receptor downregulation of (Puighermanal et al., 2013). Also, the ability of THC administration to impair the expression of CB_1 receptors in the developing brain has been show (Tortoriello et al., 2014), although in a wider embryonic exposure time window. Therefore, we examined CB_1 receptor expression after our subchronic THC exposure paradigm. We found CB_1 receptor levels significantly downregulated in THC-treated embryonic brains when compared to controls by western blot (Fig. 5 A, B). We also examined CB_1 expression in postnatal brains by immunofluorescence. Quite unexpectedly, we found a substantial decrease in CB_1 receptors presence in the cerebral cortex by P15, indicating that CB_1 downregulation is perdurable in the developing cortex upon repeated THC exposure. Overall, these findings might provide evidence at the molecular level of the functional consequences in the embryonically THC-administered offspring, that recapitulates the neurodevelopmental alterations induced by genetic CB_1 receptor ablation.

Increased seizure susceptibility in THC-exposed mice

Forebrain development alterations are a common cause of the aberrant configuration of neuronal circuitry underlying epileptic-like pathologies (Sisodiya, 2004). We were curious about whether

altered subcerebral projection neurons ensued by cannabinoid treatment presumably associated to other cortical development alterations resulted in an increased seizure susceptibility. To explore this possibility we administered a series of subconvulsive doses of pentylenetetrazol (PTZ) to four experimental groups: WT vehicle, WT THC, $CB_1^{-/-}$ vehicle and $CB_1^{-/-}$ THC. PTZ doses (30mg/Kg, i.p.) were injected until the onset of generalized tonic-clonic seizures was reached in each animal, (Manent, Wang, Chang, Paramasivam, & LoTurco, 2009). Importantly, seizure threshold was significantly lower in CB_1 knockouts than in their WT littermates, and no effect of embryonic cannabinoid exposure was found in these mice. However, in WTs, THC exposure during prenatal development significantly decreased PTZ-induced seizure threshold (Fig. 4).

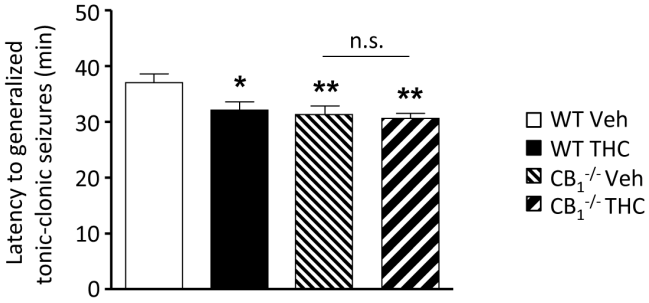

Figure 5. Decreased PTZ-induced seizure threshold in THC-exposed mice. THC- and vehicle-treated mice were given serial injections of the pro-convulsive drug pentylenetetrazole (PTZ, 30mg/Kg) every 10 minutes until generalized tonic-clonic seizures were observed. Time elapsed since the first injection was calculated and compared among groups. n= 6-10 mice per group. *,p<0,05; **, p<0,01.

DISCUSSION

Our results show that THC exposure during prenatal development negatively impacts subcerebral projection neuron development and, therefore, their function in the mature brain. Overall, our data reflect that THC acted as a functional antagonist of CB_1 signaling, even at relatively low doses used for this study. Therefore, the narrow temporal window of THC-mediated CB_1 functional alterations in our experimental paradigm might represent a plausible model of a time-restricted CB_1 loss of function, thereby further confirming the developmentally-specific role of CB_1 receptors in the refinement of corticospinal connectivity, previously reported using full and Glu-CB_1 knockouts (Diaz-Alonso et al., 2012b). These findings might be relevant from a clinical perspective, as growing evidence links abnormal cortical development with the onset of upper motor neuron diseases, such as ALS (Ozdinler et al., 2011)

Epilepsy is one of the most common neurological conditions, affecting nearly 3% of the population. This disorder can emerge as a consequence of multiple factors, including traumatic injury, brain tumors, CNS infections and cerebrovascular pathologies (Dichter, 2009). Also, in many cases, epilepsy appears as a consequence of the aberrant configuration of neural circuitry during development (Sisodiya, 2004). PTZ susceptibility was previously shown to be reduced upon the application of cannabinoid agonists, while increased by pharmacologically blocking CB_1 signaling (Shafaroodi et al., 2004). Here we present data causally linking alterations in the developmental role of endocannabinoid signaling by THC exposure with an epileptogenic neural circuitry configuration. Alterations in CNS development beyond CSMN generation and /or specification following THC administration are likely to contribute to the observed increased PTZ susceptibility. In that sense, CB_1 expression alterations lasting in the postnatal might also reflect a change in the synaptic arrangement in the cortex, probably reflecting alterations also in the development of neuronal populations other than pyramidal cells. Hence, further investigation is required to underscore the relevance of other neuronal lineages targeted by THC in the behavioral consequences of prenatal cannabinoid exposure.

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3.3. **CHAPTER 3.** Characterization of the pro-migratory role of CB_1 receptor signaling in developing pyramidal neurons.

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The CB_1 cannabinoid receptor signals radial migration of pyramidal neurons in the developing mouse cortex through the inhibition of RhoA

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Cortical pyramidal neurons are born in the ventricular and subventricular zones. Then, they migrate radially towards the developing cortical plate along the radial glial scaffold. The appropriate formation of the stereotypical 6-layered mature mammalian neocortex critically depends on the proper migration of newborn pyramidal cells, as exemplified by the diverse malformations found in the mature cortex as a consequence of alterations of this process. However, our knowledge about the mechanisms that link extrinsic signals to the activity of the cell-autonomous factors that ultimately trigger the pro-migratory molecular cascade is still incomplete. Endocannabinoids, acting retrogradely via their CB_1 receptors, are well known neuromodulators in the adult brain and also exert a neurodevelopmental regulatory role, tuning several processes such as progenitor cell proliferation and identity, neural specification and morphogenesis. In addition, it has been suggested that the endocannabinoid signaling regulates neuronal migration, but the precise molecular mechanisms implicated remain unclear.

In this study we investigated the potential chemoattractant profile of the endocannabinoid 2-arachidonoylglicerol (2-AG) for newborn pyramidal cells. Using embryonic cortical explants we show that neuronal migration is favoured towards a source of 2-AG compared with the corresponding control. To explore the cell-autonomous role of CB_1 receptors in the control of radial migration we performed *in utero* electroporation-mediated CB_1 knockdown in dorsal telencephalic progenitor cells. Delayed radial migration of siRNA-CB_1-electroporated cells was consistently observed when compared to siControl-transfected cells. Interestingly, CB_1 signaling was found to promote radial migration in different pyramidal neurogenic waves. Moreover, we found that CB_1-dependent regulation of radial migration was shown to be independent of CB_1-mediated progenitor cell proliferation. Finally, the migration deficits induced by CB_1 loss of function were rescued by concomitant silencing the small G protein RhoA or coexpression a non-phosphorylatable form of cofilin. Overall, our results show that endocannabinoid signaling through CB_1 receptors drive radial migration in the developing mouse cortex, and identify the involvement of RhoA and downstream regulation of actin cytoskeleton dynamics as key mediators of CB_1 receptor pro-migratory signaling.

INTRODUCTION

During neuronal development diffentiating cells undergo a migration process after (or concomitantly) they exit cell cycle. Therefore, neuronal migration essentially contributes to the construction of the refined cytoarchitectonic harmony of the cerebral cortex (Marin, Valiente et al. 2010). Not surprisingly, gross abnormalities in cortical organization are ensued by disruptions of this process, with devastating consequences in the adult brain function, including mental retardation, epilepsy and mood disorders (Valiente and Marin 2010). In the developing cerebral cortex two different modes of neuronal migration coexist: by one side, cortical excitatory neurons, which are born in the proliferative niche of the lateral ventricles' dorsal wall, migrate radially towards the cortical plate to finally rest in their corresponding, birthdate-codified, cortical layer (Noctor, Martinez-Cerdeno et al. 2004). On the other hand, cortical GABAergic interneurons arise from the medial and caudal ganglionic eminences and they undergo a longer tangential journey to settle in their final position in the cortex

(Marin 2013). Neuronal migration depends on the dynamic rearrangement of the cytoskeleton. Cytosketal plasticity is controlled by a plethora of regulatory proteins, which activity relies in the concerted action of intrinsic (cell-autonomous) and extrinsic factors. Among the former, a prominent role has been assigned to the proneural transcription factors Ngn2 and Ascl1 which, by modulating the small GTP-binding proteins Rnd2 (Heng, Nguyen et al. 2008) and Rnd3 (Pacary, Heng et al. 2011), promote radial migration in distinct phases. Among the latter, the glycosylated extracellular matrix-associated protein Reelin, acting through the ApoER2 receptor, plays an essential role during radial migration, as reflected by the impairment its deficiency causes in the formation of the stereotypical six-layered neocortex (Hashimoto-Torii, Torii et al. 2008). Also BDNF and semaphorins (Chen, Sima et al. 2008), among others, have been shown to regulate radial migration. Interestingly, how the extracellular signals are transduced to the intrinsic regulators to tune radial migration is beginning to be understood; for instance recent work has shown how semaphorin signaling, acting through PlexinB2 receptors, modulates RhoA signaling to favor a pro-migratory actin cytoskeleton configuration (Azzarelli, Pacary et al. 2014).

Besides its well-known neuromodulatory role at adult synapses (Kano, Ohno-Shosaku et al. 2009), the endocannabinoid system plays a pleiotropic role in the regulation of several processes during CNS development. CB_1 cannabinoid receptors are expressed in the developing cerebral cortex from early stages, and have been shown to control the proliferation (Aguado, Monory et al. 2005) and identity of cortical neural precursor cells (Diaz-Alonso, Aguado et al. 2014), the balance of neuronal and glial cell generation (Aguado, Palazuelos et al. 2006), the specification of pyramidal neurons (Diaz-Alonso, Aguado et al. 2012), and the regulation of neuronal morphogenesis and axon guidance (Berghuis, Rajnicek et al. 2007, Keimpema, Barabas et al. 2010). CB_1 receptors have been shown to play a modulatory role in neuronal migration in the developing brain, both in the excitatory neurons' radial migration towards their appropriate cortical layer (Mulder, Aguado et al. 2008) and in the colonization of the hippocampus by GABAergic interneurons (Berghuis, Dobszay et al. 2005). CB_1 receptors are physiologically engaged by lipidic mediators, namely endocannabinoids, of which 2-AG and anandamide are the best known members. Blockade of endocannabinoid breakdown has been shown to increase the rate of radial migration, whereas its enhancement leads to the complementary phenotype (Mulder, Aguado et al. 2008). Of note, endocannabinoid signaling potentiation also promotes migration of newborn neurons along the rostral migratory stream in the postnatal mouse brain (Oudin, Gajendra et al. 2011). However, evidence supporting the modulatory role of the endocannabinoid system in radial migration *in vivo* is lacking, and the mechanisms triggered by CB_1 receptors to promote neuronal migration remain poorly understood.

A wide variety of neurodevelopmental diseases are caused by the disruption of neuronal migration. Understanding of the biological mechanisms responsible of finely tuned corticogenesis emerge as a key requisite for the elaboration of rational therapeutic interventions aimed at ameliorating malformations of cortical development responsible of epileptogenesis and neuropsychiatric disorders. Noteworthy, a genetic origin has been identified for a majority of human diseases caused by neuronal migration alterations, and the identification of the genes disrupted in such diseases, in most cases codifying for cytoskeletal or cytoskeleton regulatory proteins, has boosted the basic research on this topic (Barkovich, Guerrini et al. 2012). In this scenario, results shown here indicate that CB_1 receptors act as a novel signaling platform, able to sense extracellular lipidic signaling cues promoting neuronal radial migration in vivo. These findings might have implications in human brain developmental alterations originated by deficits in neuronal migration.

MATERIALS AND METHODS

Materials

The following materials were kindly donated: DAGLα -GFP construct (Dr Patrick Doherty, King's College, London, UK) and $CB_1^{-/-}$ colony-founding mice (Prof B. Lutz, Johannes Guttenberg University, Mainz, Germany).

Animals

Experimental designs and procedures were approved by the Complutense University Animal Research Committee in accordance with the European Commission regulations. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Mice were maintained in standard conditions, keeping littermates grouped in breeding cages, at a constant temperature (20±2ºC) on a 12-h light/dark cycle with food and water *ad libitum*. The generation and genotyping of $CB_1^{-/-}$ and wild-type (WT) littermates has been reported

elsewhere and was performed accordingly (Monory, Massa et al. 2006). Mouse embryonic tissues were obtained upon timed mating as assessed by vaginal plug observation (E0.5).

Immunofluorescence and confocal microscopy

Cell proliferation was determined after intraperitoneal iodo- and bromo-deoxyuridine (IdU, BrdU) injection (100 µg/g body weight) of pregnant females at E13.5 and E16.5 as indicated. Coronal embryonic and postnatal brain slices (14 and 30 µm-thick, respectively) were processed as previously described (Diaz-Alonso, Aguado et al. 2012). Cortical layers were identified by their discrete cell densities as visualized by DAPI counterstaining. Immunofluorescence was performed, after blockade with 5% goat serum, by overnight incubation at 4ºC with the indicated primary antibodies, followed by incubation for 1 h at room temperature with secondary antibodies. The appropriate highly cross-adsorbed AlexaFluor secondary antibodies (Invitrogen, Carlsbad, CA) were used. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with 2 passes by Kalman filter and a 1024X1024 collection box. Immunofluorescence data were obtained in a blinded manner by an independent observer and all quantifications were obtained from a minimum of 6 sections from 1-in-6 series per mice. In CB_1 receptor knockdown experiments by *in utero* electroporation, cells positive for the indicated markers were quantified within the GFP^+ cell population.

***In utero* electroporation**

In CB_1 knockdown experiments, siCB_1 and siControl (Thermo Scientific) or sh CB_1 or shControl (Origene) were electroporated together with pCAG-GFP and Fast Green in the lateral ventricle of E13.5 or E14.5 embryos as described (Diaz-Alonso, Aguado et al. 2012). For CB_1 overexpression, a pCAG- CB_1-GFP construct was employed. For CB_1 ablation experiments in $CB_1^{f/f}$ embryos, a pCAG-cre-GFP vector (Addgene) was employed. Rescue experiments included the coexpression of either an shRNA directed against RhoA (Origene) or a pCAG-cofilin^{S3A} (Pacary, Heng et al. 2011). *In utero* electroporated embryos were analyzed 3 days later, or at postnatal days 2 or 10.

Explant migration assays

Cortical explants (300um^2) were prepared from E14.5 embryonic brains using a McIlwain tissue chopper and then cultured in a matrigel tridimensional structure. pCAG-DAGLα -GFP- or pCAG-GFP-transfected P19 mouse embryonic carcinoma cells were cultured as hanging drops before placing the resulting cell aggregate in front of the corresponding explant. Co-cultures were maintained in neurobasal medial, supplemented with N2 (Milipore) and B-27 (Invitrogen) for 18h and then fixed. Cell migration from the explants was analized in 4 quadrants, and the proximal/distal ratio (with respect to the corresponding cell aggregate) was calculated.

Data analyses and statistics

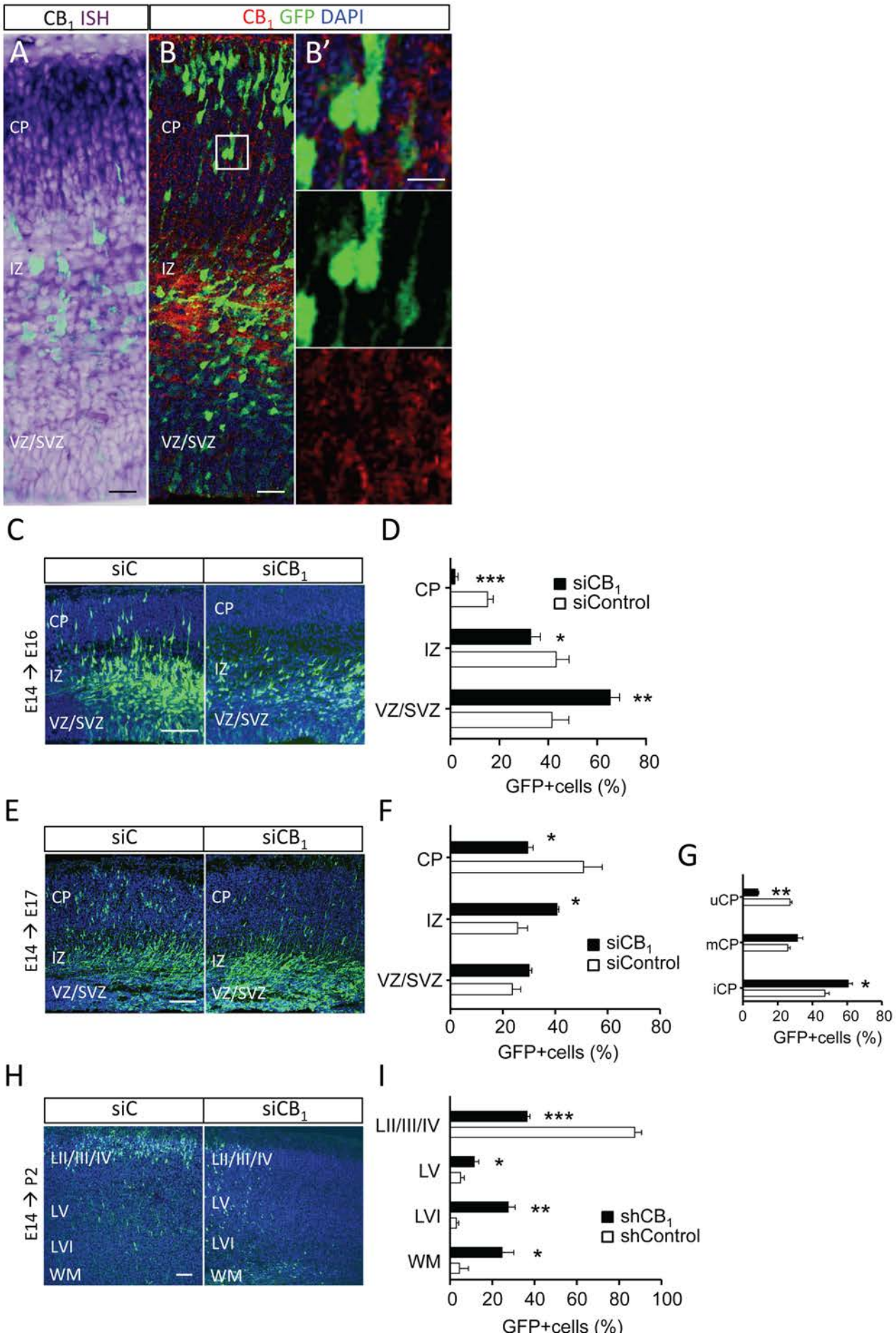
Results shown represent the means ± S.E.M., and the number of experiments is indicated in every case. Statistical analysis was performed by one- or two-way ANOVA, as appropriate. A *post-hoc* analysis was made by the Student-Neuman-Keuls test.

RESULTS

Acute CB_1 cannabinoid receptor knockdown in vivo interferes with Radial migration

CB_1 receptor expression in the developing cortex has been studied in detail. There is a broad consensus in the field on the existence of discrete CB_1 expression domains: low CB_1 expression levels are found in cortical progenitor cells (Diaz-Alonso, Aguado et al. 2014), and increasing expression levels accompany neuronal differentiation (Gaffuri, Ladarre et al. 2012). We first approached the analysis of the expression of the CB_1 receptor in radially migrating neuroblasts by *in situ* hybridization and immunofluorescence detection of the CB_1 receptor in radially migrating GFP-labeled cells after *in utero*-electroporation (Fig. 1A). We next wanted to determine the functional

Figure 1. The CB_1 receptor is expressed in newborn pyramidal neurons and signals radial migration. (A) Representative CB_1 ISH images in E16.5 developing cortex, electroporated with GFP at E14.5. (B, B') Representative image of CB_1 receptor immunoreactivity in E17.5 cortical sections, electroporated with GFP at E14.5. Cell nuclei were counterstained with DAPI. Inset of the indicated areas is shown. (C, D) Representative images and quantification of GFP^+ cells, electroporated with siCB_1 or siControl at E14.5, along the apico-basal axis of the developing cortex at E16, divided into 3 discrete regions: VZ/SVZ, IZ and CP. (E-G) Representative images and quantification of GFP^+ cells at E17, electroporated with si CB_1 or siControl at E14.5. In G, GFP^+ cell relative position within the cortical plate was also calculated. (H, I) Representative images and quantification of GFP^+ cell distribution in each cortical layer at P2. IUE were performed with an shRNA against CB_1 or an shRNA control together with a GFP expression vector at E14.5.CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone; iCP, inner CP; mCP, medial CP; uCP, upper CP; WM, white matter. Scale bars: A, B, 50mm and 20mm (insets); C, E, H, 100mm. n= at least 3 embryos/pups per group.*, $p<0.05$; **, $p<0.01$; ***,


CB1 ISH
CB1 GFP DAPI
A
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B'
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IZ
VZ/SVZ
C
siC
siCB1
E14 → E16
D
siCB1
siControl
GFP+cells (%)
E
E14 → E17
F
G
uCP
mCP
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H
E14 → P2
LII/III/IV
LV
LVI
WM
I
shCB1
shControl

relevance of cell-autonomous CB_1 receptor signaling in the regulation of radial migration *in vivo*. To approach this, we acutely knocked down CB_1 receptors in radially migrating neuroblasts by *in utero* electroporation (IUE) of an siRNA directed against the CB_1 mRNA together with a GFP expression construct in E14.5, analyzing the distribution of radially migrated cells by E16.5 and E17.5. siCB_1-mediated knockdown efficacy had previously been determined in over 50% CB_1 expression reduction (Diaz-Alonso, Aguado et al. 2014). As shown in Fig. 1B-G, CB_1 loss-of-function reduced newborn pyramidal cell migration. Therefore, siCB_1-GFP$^+$ cells (right hand panels, black bars) remained clustered in a retarded position along the developing cortical parenchyma when compared to the siControl-GFP$^+$ cells (left hand panels, white columns). Specially, both after 2 days *in utero* (DIU) and 3DIU we observed an apparent blockade of the invasion of the cortical plate (CP) in CB_1 knocked-down cells that, instead, appear to be retained in the intermediate zone (IZ). The majority of siCB_1-GFP$^+$ cells were not able to cross the IZ and enter in the CP. Moreover, when we restricted our analysis only to those cells found in the CP, we also found a significant delay in CB_1 receptor knockdown cells, that were less abundant in upper cortical layers of the cortex where, given the timing of the electroporation –E14.5-, these cells are committed to migrate (Fig.1G). To exclude the possibility that CB_1 receptor knockdown cells suffer a transient delay in their radial migration, but they can finally reach their final positions if given enough time, we extended our *in utero* electroporation experiments allowing embryos to develop until an early postnatal stage (P2). In these experiments the migration deficit observed upon CB_1 expression manipulation was still observed, (Fig. 1H, I), and confirmed that acute CB_1 loss of function compromises radial migration of newborn neurons in the developing cortex.

The definitive confirmation of these results came from IUE experiments in which embryos were allowed to develop until postnatal day 10 (P10). Whereas we observed more GFP$^+$ cells in the upper layers of sh CB_1-electroporated cortices,we consistently found GFP$^+$ cells retarded in deep layers V/VI and, strikingly, we even found clusters of GFP$^+$ cells stacked in the white matter in CB_1 knock-down experiments (Fig. 2 A, B). We then analyzed the identity of neuronal CB_1 receptor-knockdown cells. Importantly, CB_1 receptor knocked-down cells located at delayed positions in the cerebral cortex when compared with sh-control-GFP$^+$ cells , are immunorreactive for the upper layer specification marker Satb2 (Fig. 2C). This finding indicates that specification in arrested cells is not affected, therefore ruling out that migration deficits are indirectly originated by a neuronal fate switch.

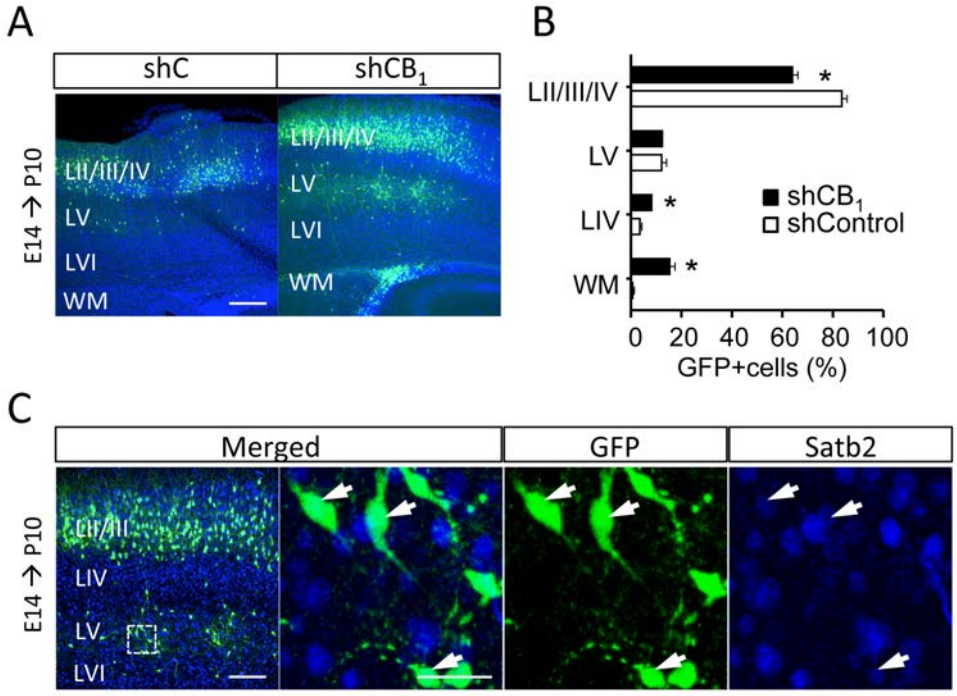

Figure 2. Radial migration deficits evoked by CB_1 loss of function are long-lasting and independent of pyramidal subtype specification. (A, B), Representative images and GFP$^+$ cell distribution of sh CB_1- and shControl-electroporated brains (E14.5), analysed at P10. The cerebral cortex is divided in 4 discrete regions, WM (white matter), LVI, LV and LII/III. (C), Immunofluorescence showing Satb2 expression in most GFP$^+$ cells retained in cortical deep and medium layers in sh CB_1 electroporated brains. WM, white matter. Scale bars: A, 200mm ; C, 100mm and 20mm (insets). n= at least 3 pups per group. *, $p<0.05$.

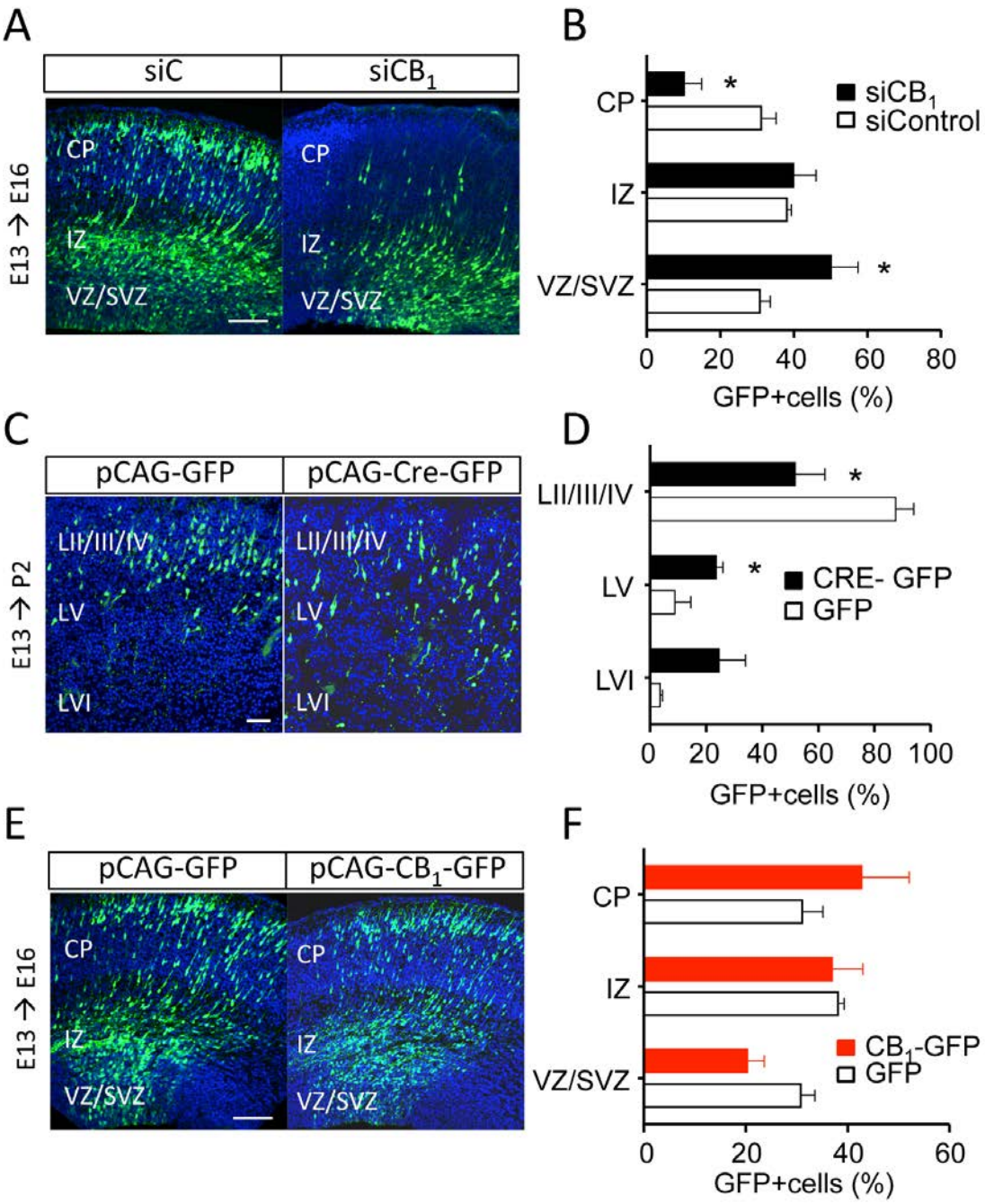

Figure 3. CB_1 signaling promotes radial migration in distinct pyramidal neurogenic waves during development. (A, B), WM, Representative images and quantification of GFP^+ cells, electroporated with si CB_1 or siControl at E13.5 to target earlier born pyramidal neurons, along the apico-basal axis of the developing cortex at E16, divided into 3 discrete regions: VZ/SVZ, IZ and CP. (C, D), Images and quantification of the distribution of GFP^+ cells, transfected in E13.5, in the distinct cortical layers by P2. (E, F), Images and analysis of the distribution of GFP^+ cells, electroporated in E13.5 with a pCAG- CB_1-GFP or a pCAG-GFP construct as a control, in the cortex by E16.5. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/ subventricular zones. Scale bars, 100mm. n= at least 3 embryos/pups per group. *, $p<0.05$.

CB_1 receptor regulation of neuronal migration is preserved in neuronal progenitors of deep layer neurons

We then asked ourselves whether the observed CB_1-dependent promotion of radial migration was specific for certain pyramidal neuron-subtypes, and thus exclusive for E14.5-born cells. To answer this, we performed a series of IUE experiments at E13.5, that were analyzed three days later, at E16.5 (Fig. 3A, B). Similarly to our previous observations targeting E14.5 cells, an overall delay in GFP^+ cell migration was observed in CB_1- knocked-down brains after 3 DIU. Delayed radial migration was still present at P2 in $CB_1^{f/f}$ neurons electroporated with a pCAG-CRE-GFP vector (Fig. 3C, D). These findings confirmed the specificity of CB_1 receptor loss of function manipulation and demonstrate that CB_1-dependent promotion of radial migration, rather than being subtype-specific, is a general mechanism in cortical pyramidal cells. In order to investigate the consequence of the overexpression of CB_1 receptors

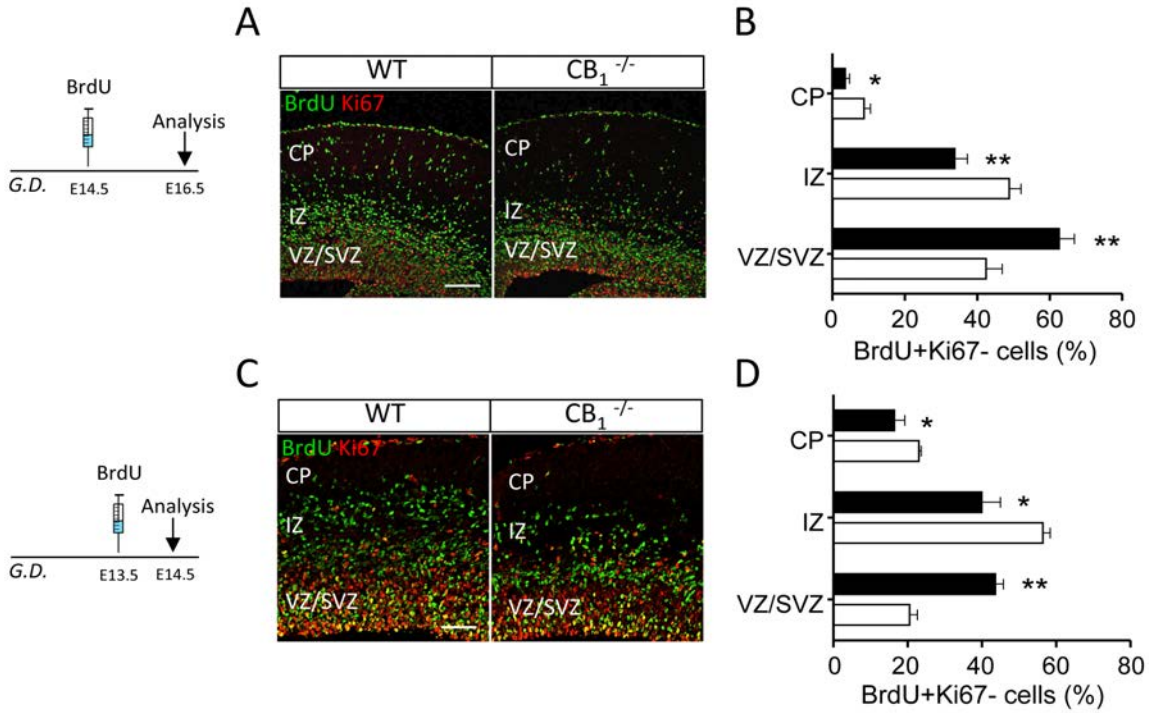

Figure 4. CB_1 signaling promotes early steps of radial migration independently of cortical progenitor proliferation. (A, B) Pregnant $CB_1^{+/-}$ female mice crossed with $CB_1^{+/-}$ male mice were injected with BrdU at E14.5 and embryonic cortices analyzed at E16. Quantification of BrdU+Ki67-/ BrdU+ cells was performed, allowing the assessment of initial steps of migration in cells having exited cell cycle within these 2 days. Representative images of are shown. (C, D) Similar experimental protocol was performed, injecting BrdU at E13.5 and performing the analysis at E14.5, to corroborate the existence of a CB_1-dependent modulation of the initial steps in migration. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zones. Scale bars, A, 100mm; C, 50mm. n= at least 4 embryos for each group. *, $p<0.05$; **$p<0.01$.

in neuronal migration, we performed electroporations with a pCAG-CB_1-GFP expression plasmid and analyzed the position of GFP^+ cells three days later, comparing them with control, pCAG-GFP transfected cells. As shown in Fig. 3E, F, CB_1 receptor overexpression had a subtle, non-statistically significant pro-migratory effect indicating that the endogenous CB_1 pro-migratory signaling during cortical development can not be overpassed by constitutive CB_1 receptor activity.

CB_1 receptor regulation of radial migration is independent of progenitor cell proliferation

CB_1 receptors have been shown to play a role in the regulation of cortical progenitor cell proliferation (Aguado, Monory et al. 2005, Diaz-Alonso, Aguado et al. 2014). Therefore, we wondered whether CB_1 LOF-evoked alteration of cortical progenitor cell cycle could account for the observed impact in radial migration. To investigate this possibility, we performed birthdate labeling experiments with BrdU (E14.5) followed by immunofluorescent detection of Ki67 (E16.5) in order to discard cells that were still proliferating, in CB_1 knockout embryos and control littermates. As shown in Fig. 4A, B, the fraction of $BrdU^+Ki67^-$ cells, namely the cells that had exited cell cycle between E14.5 and E16.5, was significantly retarded in their radial migration in $CB_1^{-/-}$ embryos when compared to their WT littermates. We also performed a shorter experiment, in which we administered BrdU to pregnant females by E13.5, analyzing the samples one day after, E14.5. Again, we identified a significant delay in the earliest steps of migration in the absence of CB_1 receptor signaling (Fig. 4C, D). In summary, these experiments suggest that the first steps of pyramidal neuron migration towards the cortical plate require CB_1 signaling, independently of CB_1 regulation of pyramidal precursor cell proliferation.

The endocannabinoid 2-AG acts as a chemoattractant for migrating newborn pyramidal neurons

Many GPCRs including CB_1 have a constitutive/tonic signaling, independently of the binding of their ligand (Gaffuri, Ladarre et al. 2012). This raises the question of whether CB_1-dependent promotion of neuronal migration in the developing brain requires a dynamically regulated source of endocannabinoid ligands. To explore this issue, we prepared embryonic

cortical explants and challenged them with a source of endocannabinoids. Migration of neurons from these explants showed a marked preference to occur towards a cluster of P19 cells transfected with the 2-AG synthesizing enzyme DAGLα, when compared with control, GFP-transfected cells (Fig. 5A, B), thus pointing to 2-AG as a chemoattractive molecule for radially migrating neuroblasts.

Rho A and cofilin loss of function rescue CB_1 receptor knockdown-induced radial migration arrest

Various neuronal migration-regulating mechanisms converge in the modulation of the RhoA activity (Cappello, Bohringer et al. 2012, Pacary, Azzarelli et al. 2013, Azzarelli, Pacary et al. 2014) and, given that previous evidence point to a mechanistic link between CB_1 receptor signaling and RhoA (Berghuis,

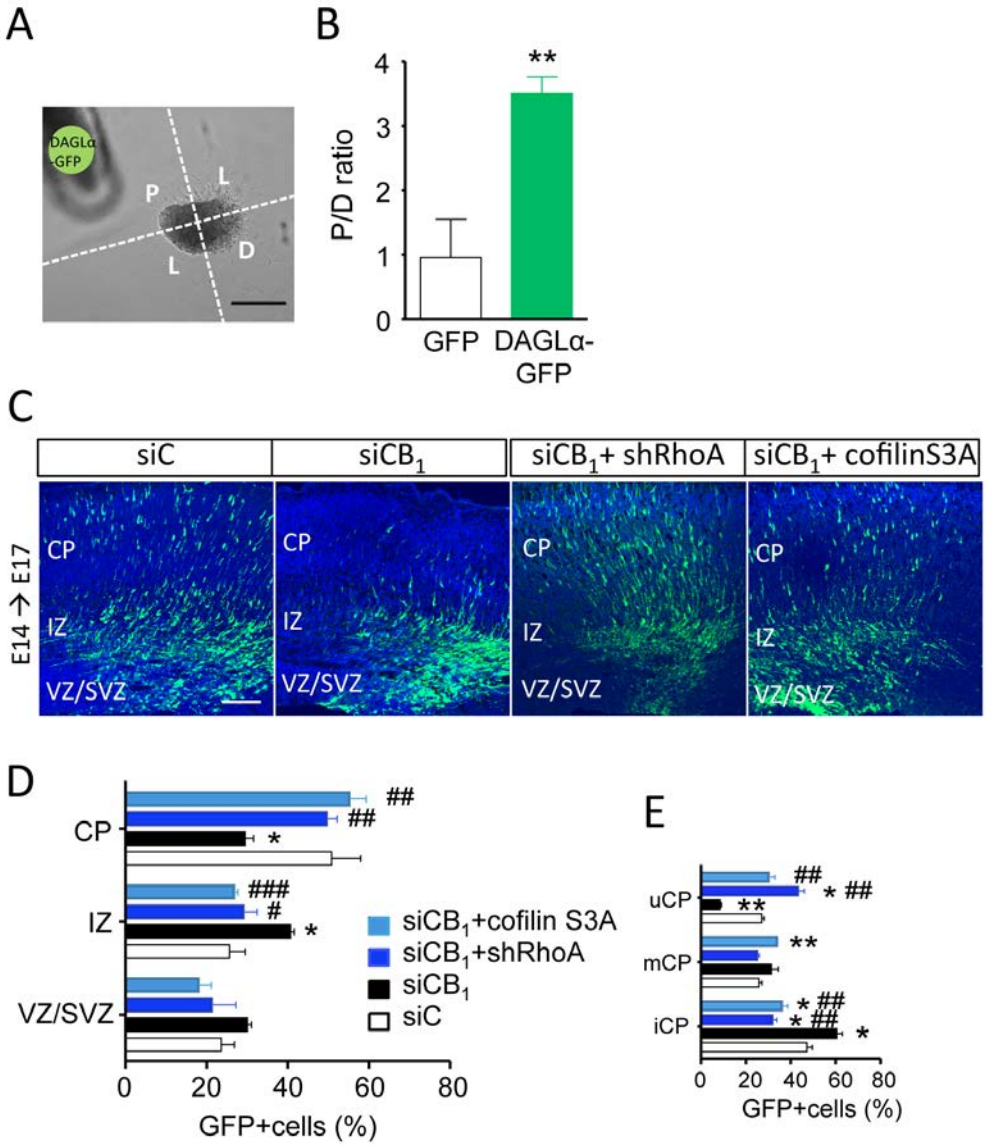

Figure 5. The endocannabinoid system promotes radial migration via the modulation of actin cytoskeleton dynamics. (A, B), Cortical explant (300mm2) cultures were prepared and cultured in a tridimensional matrix, facing them to DAGLα - or GFP-transfected P19 cells. Migration towards the source of 2-AG (or control cells) was quantified after 18 h as the P (proximal) / D (distal) ratio. At least 5 different explants from 3 different embryos were analyzed per condition. (C-E), Representative images and quantifications of in IUE experiments in which the siRNA against CB_1 was coelectroporated either with an shRNA against RhoA (dark blue bars) or a non-phosphorylatable mutant of cofilin (cofilin S3A, light blue bars). Distribution of GFP^+ cells was assessed in the different cortical compartments (D). Also, CP-migrated cells relative position was quantified in 3 bins, as in Fig. 1 (E). n= at least 3 embryos/pups per group. CP, cortical plate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone; iCP, inner CP; mCP, medial CP; uCP, upper CP; P, proximal; L, lateral; D, distal. Scale bars: A, 25mm; C, 100mm. *, $p<0.05$; **, $p<0.01$; #, $p<0.05$ vs $siCB_1$; ##, $p<0.01$ vs $siCB_1$; ###, $p<0.001$ vs $siCB_1$

Rajnicek et al. 2007), we hypothesized that the pro-migratory effect of CB_1 receptors in newborn pyramidal cells could rely on the modulation of this pathway. As RhoA expression by newborn pyramidal neurons has recently been shown to be largely dispensable for their migration towards the cortex, while its overactivation results in radial migration arrest (Cappello, Bohringer et al. 2012) (Pacary, Heng et al. 2011), we downregulated RhoA in an attempt to rescue the migration deficits observed in CB_1-knockdown cells. We coelectroporated an shRNA directed against RhoA (Pacary, Heng et al. 2011) together with the si CB_1. As shown in Fig. 5C-E, knocking down RhoA fully rescued CB_1 loss of function-induced migration arrest, and restored both the distribution of GFP^+ cells along the different cortical compartments (VZ/SVZ, IZ and CP) and the position of cells within the cortical plate (Fig 5E). Moreover, we also tested the ability of a non-phosphorylatable form of cofilin, that prevents F-actin to excessively depolymerize (cofilin S3A, (Pacary, Heng et al. 2011)) to rescue CB_1 loss of function-derived migration arrest. As shown in Fig. 5A-C, this intervention was also successful. These results, thus demonstrate that CB_1 signaling contributes to the fine regulation of actin cytoskeleton remodeling machinery that, in turn, drives radial migration of projection neurons in the developing cerebral cortex.

DISCUSSION

The results presented here show, for the first time, that acute CB_1 cannabinoid receptor loss of function impairs radial migration of newborn pyramidal neurons in the cerebral cortex *in vivo*. We demonstrate that CB_1-dependent regulation of radial migration operates in different subsets of pyramidal cells and in a pyramidal precursor cell-independent manner. Our results point to a prominent role of CB_1 receptor signaling in the promotion of pyramidal neuron progression through the cortical plate, as the IZ-CP transition seems to be the most affected step in radially migrating neuroblasts devoid of CB_1. We also provide a plausible molecular logic to explain the mechanism of action of CB_1 signaling during pyramidal neuronal radial migration in the developing cortex, including i) the apparent chemotactic profile of its ligand, the endocannabinoid 2-AG, for newborn cortical pyramidal neurons and ii) the identification of RhoA inhibition as a putative downstream effect of CB_1 signaling during radial migration. In our working model, in the absence of CB_1 RhoA signaling would become de-inhibited and result in excessive actin cytoskeleton turnover, as suggested by the fact that artificially promoting stable actin filaments via the expression of a non-phosphorylatable form of cofilin fully rescues migration defect when CB_1 function is lost.

Radial migration is largely dependent on the dynamic regulation of the cytoskeleton (Valiente and Marin 2010). Remarkably, actin cytoskeleton remodeling plays a fundamental role in this process, and RhoA-mediated tuning of the adequate balance between F-actin polymerization and depolymerization seems to be crucial for the correct rate of migration, as both gain- and loss-of-function result in radial migration arrest (Pacary, Heng et al. 2011) (Azzarelli, Pacary et al. 2014). Interestingly, our results are in agreement with the reported role for RhoA in migration, that is, permissive at normal levels, but deleterious when overactive (Cappello, Bohringer et al. 2012). Our results also reproduce the slightly "over-migration" phenotype observed in the upper cortical layers by Cappello *et al*. in RhoA loss of function conditions, suggesting that RhoA also acts through other mechanisms.

In summary, endocannabinoids add to the panoply of extracellular cues previously shown to play a role in the synchronization of the different steps that guarantee the appropriate migration of newborn neurons in the cortex (Sobeih and Corfas 2002) (Chen, Sima et al. 2008, Azzarelli, Pacary et al. 2014). Many mechanistic aspects of CB_1-mediated promotion of radial migration will require future efforts to be fully understood. The potential implication of the malfunction of the mechanism described here in the occurrence of neurodevelopmental pathologies with a migration base is an exciting perspective for future research. Also, the possible implication of this signaling system in the regulation of the migration of the other main neuronal population of the cerebral cortex, namely GABAergic interneurons, remains as an open question waiting for future research to be elucidated.

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4. DISCUSSION

CB_1 receptors present in cortical progenitor cells control their identity the self-renewal/ neurogenesis balance

CB_1 receptor expression by neural stem and progenitor cells has been a matter of debate for years, and contrasting results have been reported in different laboratories (Mulder et al., 2008; Vitalis et al., 2008). Here we have shown that CB_1 cannabinoid receptors are present, albeit at low levels, in the proliferative compartments of the developing dorsal telencephalon **(Chapter I, (Diaz-Alonso et al., 2014))**. Despite their low level of expression, CB_1 receptors participate, at least partially in a cell-autonomous manner, in the control of the activity of Pax6, and therefore in the identity and behavior or radial glial cells (Heins et al., 2002; Osumi et al., 2008). Our results suggest that CB_1 signaling is specifically engaged in the regulation of the cell division mode, hence in the identity of daughter cells. CB_1 receptor loss of function results in a biased outcome of radial glial cell divisions, favoring direct neurogenesis instead of the indirect, amplifying neurogenesis through the generation of intermediate progenitors. As a consequence, a transiently increased cortical plate thickness is detected in CB_1-deficient embryos at the expense of the cortical progenitor-populated VZ and SVZ (data not shown). Interestingly, the physiological relevance of CB_1 receptor signaling is not always directly related to its abundance. To note, despite it is expressed 10-20 times at higher levels in GABAergic neurons than in excitatory neurons (Marsicano and Lutz, 1999; Monory et al., 2006; Puighermanal et al., 2013), CB_1 receptor seems to be more efficiently coupled to intracellular effectors in glutamatergic cells (Steindel et al., 2013). The advent of conditional CB_1 knockout mouse lines (Monory et al., 2006) confirmed the involvement of glutamatergic CB_1 in a plethora of endocannabinoid system-mediated functions, as well as in some of the effects of THC administration (Ramikie et al., 2014; Soria-Gomez et al., 2014).

These precedents, together with the functional evidences for a cell-autonomous role of CB_1 receptors we have described in this study, indicate that the low levels of CB_1 receptors found in cortical progenitors are efficiently coupled to intracellular effectors, to tune cortical progenitor self-renewal and neurogenesis. Nevertheless, we cannot completely rule out the participation of CB_1 receptors expressed by postmitotic neurons in some of the CB_1-dependent functions in cortical progenitors shown here. In that sense, given that CB_1 receptor expression is notably increased throughout the neuronal differentiation process, it is plausible that its signaling influences cortical progenitor behavior through the induction of specific diffusible ligands by differentiating cells. This feedback mechanism has been shown play a role, for instance, in the assymetric inheritance of the ubiquitin

ligase Mib1, required for functional Notch signaling in the apical basal daughter cells, committed to self-renew and differente, respectively (Dong et al., 2012; Franco and Muller, 2013).

The involvement of GPCRs in the regulation of the mitotic spindle orientation and, therefore, in the outcome of cell divisions has been reported to specifically depend on the β/γ subunits of the G-protein they activate (Sanada and Tsai, 2005). Noteworthy, signaling from CB_1 receptors has been shown to rely in part by β/γ subunits for some of their physiological roles, such as ion channel conductance modification and MAPK pathway activation (Bouaboula et al., 1995; Howlett, 2005; Kreitzer and Regehr, 2001). Future research might indicate whether CB_1-dependent tuning of cortical progenitor mode of division is also mediated by this mechanism.

CB_1 receptor signaling recruits mTORC1 activity to tune cortical progenitor cell identity and proliferation

Our results point to the mTORC1 pathway activation as a key step in the CB_1 signaling-dependent modulation of the gene expression machinery in radial glial cells and, therefore, in the balance between self-renewal and generation of intermediate progenitors and the direct generation of neurons. These findings are in contrast with previous reports ruling out the contribution of the mTORC1 pathway in the mediation of cannabinoid receptors pro-proliferative effect on neural progenitors from the adult hippocampus (Jiang et al., 2005). However, it is conceivable that the mere distinct origin of the neural precursors assayed in both cases accounts for the different mechanisms found to be involved. Noteworthy, our work is the first to our knowledge in assigning a role to the mTORC1 route in the transcriptional activity of the master transcriptional regulator Pax6. This finding has potential implications beyond endocannabinoid signaling as, conceivably, more niche-derived cues activate intracellular signaling cascades that converge in this mechanism (Zoncu et al., 2011). The mechanism described here could also apply in other cellular contexts. Other groups have reported a pivotal role of the mTORC1 pathway in the regulation of quiescence *versus* proliferation in adult neurogenic niches (Paliouras et al., 2012). Cannabinoid receptors signal progenitor cell proliferation in the adult brain, both physiologically and upon excitotoxic insult (Aguado et al., 2005; Aguado et al., 2007; Jin et al., 2004; Palazuelos et al., 2006). Interestingly, we also showed that CB_2 cannabinoid receptor-driven proliferation relies in the activation of the mTORC1 signaling cascade in the adult dentate gyrus (Palazuelos et al., 2012). Hence, endocannabinoid signaling-mediated mTORC1 activity could act as a homeostatic mechanism regulating adult neurogenesis rate, triggering the neurogenic adult niche responses upon traumatic, age- o disease- related challenges by recruiting mTORC1 activity (van Wijngaarden and Franklin, 2013).

This regulatory function of cannabinoid receptors over adult neurogenesis may have interesting applications under a clinical perspective. Notably, disruption of adult neurogenesis is causally linked to several human neuropsychiatric conditions including, but not limited to, stress, anxiety and major depression (Kempermann et al., 2004). Also, a gradual decrease in adult neurogenesis accompanies age-related cognitive decline (Kempermann, 2012). Interestingly from a therapeutic point of view, CB_1 cannabinoid receptor signaling activation has anxiolytic effects that rely in the enhancement of adult neurogenesis (Jiang et al., 2005). CB_1 receptor was shown to be also required for the proneurogenic effect of the non-psychoactive- phytocannabinoid cannabidiol (CBD), although this cannabinoid acts through several molecular/cellular targets (Wolf et al., 2010). Particularly, we showed that CB_1-dependent enhancement of adult neurogenesis induced by CBD treatment underlies its anxiolytic profile (Campos et al., 2012), acting through a mechanism that involves the inhibition of the enzyme FAAH and, therefore increasing endocannabinoid signaling via CB_1 receptors (Campos et al., 2013).

CB_1 signaling is required for the proper generation and specification of subcerebral projection neurons

Previous evidence had ascribed an important role to CB_1 receptors in the development of cortical projection neurons (Mulder et al., 2008; Watson et al., 2008; Wu et al., 2010). In these studies, a major function of CB_1 receptors in the control of corticofugal axonal projections, especially in their fasciculation, was first characterized. Furthermore, CB_1 function later on in development, when synaptic refinement occurs, had also been described in the somatosensory cortex (Keimpema et al., 2010; Li et al., 2009).

Our results indicate that the endocannabinoid system is required specifically for the appropriate generation and specification of deep layer subcortically projecting pyramidal neurons **(Chapter II; (Diaz-Alonso et al., 2012a))**. Our work clearly indicates that CB_1 acts by tuning the activity of specific transcription factors function governing subcerebral *versus* callosal projection neuron development. Specifically, in the absence of CB_1 receptor signaling we identified an increase of Satb2-mediated Ctip2 expression, resulting in an impairment of the transcriptional regulatory program responsible for CSMN specification. Of note, the axonal projection alterations found in CB_1 knockouts clearly resemble those described for Ctip2-deficient mice (Arlotta et al., 2005). These evidences, further confirmed by the complementary phenotype found in CB_1- and FAAH-deficient mice –in which endocannabinoid levels are increased- indicate that cannabinoid receptors play a previously unknown role safeguarding the establishment of correct and functional subcerebral projections during development. Therefore, skilled motor performance, largely dependent on corticospinal

connectivity, is affected by CB_1 loss of function during development. It is important to note that, given that the eCB system signaling is key in the control of brain reward systems and motivational processes (Vlachou and Panagis, 2013), a plausible explanation of our results in the skilled motor function behavioral analyses could be the reduced interest for food in $CB_1^{-/-}$ mice. Moreover, CB_1 receptors have been shown to be involved in various steps of food intake-associated behavior (Bellocchio et al., 2010; Di Marzo and Matias, 2005; Soria-Gomez et al., 2014). For this reason, we performed control measurements aimed at identifying possible differences emerging from reduced motivation in $CB_1^{-/-}$ mice, and found no statistically significant differences among groups. Therefore, despite motivational confounding factors cannot be completely ruled out, these data strongly suggest a specific impairment in corticospinal neuron-dependent skilled motor performance in the absence of CB_1 receptors.

Of note, cortical development traits elicited by THC embryonic exposure were found to affect substantially corticospinal motor neuron development and, as a consequence, fine motor control in the adulthood **(Chapter 3; in preparation)**. Our work and also other's (Tortoriello et al., 2014) suggest that THC acts as a functional antagonist at CB_1 receptors during development, causing cannabinoid receptor desensitization and downregulation and, thereby, impeding the physiological engagement of the receptor (Keimpema et al., 2011). Besides having clear implications for the understanding of the potential deleterious effect of cannabis exposure in humans, these findings constitute a confirmation of the developmentally-restricted role of CB_1 receptors in the control of corticospinal motor neuron development and function.

Considering the growing evidence pointing to the coexistence of diverse pools of dorsal telencephalic progenitor cells, partially committed to generate deep or upper layer pyramidal neurons (Franco et al., 2012; Franco and Muller, 2013; Marin and Muller, 2014), it is plausible that CB_1 expression, instead of being homogeneous, is confined to certain populations of cortical progenitors. In our study, a specific role for CB_1 receptors emerges in the control of deep layer, subcerebrally projecting pyramidal neurons. Despite we show that this control is essentially postmitotic differentiation-specific, we cannot rule out a contribution of CB_1-dependent regulation of a putative deep-layer committed radial glial cell pool. Apart from the impairment of subcerebral projection neuron development observed in CB_1-deficient mice (present study), endocannabinoid signaling controls the switch from neurogenesis to gliogenesis (Aguado et al., 2006) in the developing cortex. Again, it might be the case that endocannabinoid signaling affects preferentially certain subsets of cortical progenitor cells including astroglial-committed progenitors. In this sense, it is tempting to hypothesize that CB_1 receptor signaling-mediated regulation of the cortical progenitor pool behavior and specification of the subcerebrally-projecting pyramidal neurons are linked. In this hypothetical

scenario, CB_1 receptors signaling would be most prominent in a deep layer-committed progenitor subpopulation, and control corticospinal motor neuron development since the mitotic stage until neuronal differentiation is completed. The molecular substrate for this possibility, although remote with our present knowledge, could involve the selective induction of Pax6-activated expression of deep layer specific genes by CB_1 signaling. Notably, ER81, a gene known to contribute to the specification of layer 5 neurons (Molnar and Cheung, 2006; Rouaux and Arlotta, 2013), is a direct target of Pax6 in the developing cortex (Tuoc and Stoykova, 2008a). In this study we have characterized the positive regulation of Pax6 activity via CB_1 receptor signaling, both using a nonselective Pax6-responding reporter construct and a more specific Tbr2 promoter luciferase construct. It is conceivable, however, that CB_1 signaling modulates Pax6 activity in a target-selective manner, thereby favoring deep-/*versus* upper layer pyramidal neuron fate.

Neuronal migration in the developing brain is regulated by CB_1 receptors

Our results assign an important role to CB_1 receptors in the regulation of cortical pyramidal cell migration during development. Furthermore, we identify the regulation of actin cytoskeletal dynamics as a key downstream effector of CB_1 pro-migratory role **(Chapter 4, in preparation)**. Previous reports had already suggested that CB_1 signaling is involved in this developmental process (Mulder et al., 2008; Saez et al., 2013), but the relevance of the endogenous CB_1 signaling-induced regulation of radial migration *in vivo*, as well as the mechanisms implicated, remained elusive. Our results contribute to shed light to the understanding of how extracellular signals coming from the environment are transduced to specific cell autonomous machinery to tune radial migration of newborn pyramidal cells and, therefore, safeguard the appropriate formation of the cerebral cortex (Manent et al., 2011; Marin et al., 2010; Sobeih and Corfas, 2002). Neuronal migration is a crucial developmental step. Multiple human neurological diseases are caused by mutations in genes engaged in the regulation of neuronal migration (Liu, 2011; Valiente and Marin, 2010). Neuronal migration deficits are associated to a wide array of human neuropathologies including, among many others, mental retardation and language impairment. One of the most common consequences of migration alterations is the appearance of epileptic foci (Rakhade and Jensen, 2009). Moreover, among many other developmental alterations converging in the appearance of an epileptogenic neuronal connectivity, neuronal migration disorders are probably the most prominent (Guerrini and Parrini, 2010). The possible influence of endocannabinoid signaling alterations and neuronal migration deficits associated on the onset of epileptic-like events is a challenging question awaiting future investigation. Interestingly, however, we have shown that THC embryonic exposure impairs CB_1 signaling in the developing brain. In our study, THC-prenatally exposed mice show increased susceptibility to PTZ-induced epileptic seizures. Whether CB_1 loss of function-derived migration

deficits underline this effect is also intriguing and deserves future attention. Noteworthy, embryonic treatment with a synthetic cannabinoid agonist (WIN 55.212-2) has been shown to affect neuronal migration, presumably also by hijacking CB_1 receptor signaling (Saez et al., 2013).

Neuronal migration is an activity-dependent process. Recent evidence pointed to the involvement of mechanisms controlling excitability in radially migrating newborn pyramidal neurons (Bando et al., 2014). This study assigns a key pro-migratory role to the KCNK-family potassium channels. CB_1 receptors have been most studied in the context of their neuromodulatory functions in the mature brain, which rely, among other mechanisms, in the modulation of ionic channels (including potassium channels) (Kreitzer and Regehr, 2001; Mackie et al., 1995). Therefore, the potential involvement of the modulation of ion channel conductivity in the pro-migratory role of the endocannabinoid system during development is an open question that remains to be addressed.

Exacerbated mTORC1 signaling is a common feature of several human mutations that affect brain development, like focal cortical dysplasia and tuberous sclerosis complex. These mutations usually affect upstream elements of the mTORC1 pathway, like STRADalpha, TSC1, Tsc2 or Rheb which, normally, contribute to maintain the activity of this central route within the physiological levels, therefore contributing to the normal brain development (Crino, 2011). Among the neuronal development traits associated to these pathologies neuronal migration is usually affected. As a consequence, neuronal circuits in the brain are miswired and, therefore, prone to the emergence of different types of epileptic conditions (Osborne, 2010). In cortical progenitor cells, CB_1 deletion is associated to mTORC1 dysfunction (present work). Moreover, we have studied CB_1 presence in human specimens containing cortical malformations and, in agreement with previous reports (Zurolo et al., 2010), we observed a specific enrichment of CB_1 presence in mTORC1-overactive cells (not shown). Altogether, these results suggest that CB_1-dependent control over mTORC1 activity might underlie the increased seizure susceptibility we observe in CB_1-deficient and THC prenatally exposed mice.

In addition to excitatory neuronal alterations, unbalanced generation of interneuron populations contribute to developmental epilepsies (Rakhade and Jensen, 2009). As the eCB system is involved in the development and morphogenesis of inhibitory neurons (Berghuis et al., 2005; Berghuis et al., 2007), it is likely that these developmental alterations may also contribute to for the increased susceptibility to epileptogenesis.

Pathophysiological implications of endocannabinoid signaling dysfunction in malformations of cortical development

Here we have studied some of the neurodevelopmental roles of the endocannabinoid system. Our work adds to previous evidences suggesting that altered cannabinoid signaling during development can exert long-lasting consequences in adult brain function (Harkany et al., 2007; Keimpema et al., 2011). Neurodevelopmental disorders are usually ensued by subtle or severe alterations of various neurogenic processes, including neuronal generation, migration, maturation and connectivity (Pang et al., 2008). Among developmental disorders, cortical alterations constitute an important example of how embryonic deficits affect adult neurological function. As previously discussed, CB_1 receptor signaling plays a regulatory role in different neural development processes involved in these pathologies. Genetic polymorphisms of cannabinoid receptors can induce subtle changes during development by influencing CB_1 signaling (Hillard et al., 2012). Likewise, mutations of ECB-metabolizing enzymes, including degrading (FAAH, ABHD6/12, MGL) or synthesizing enzymes (NAPE-PLD, DAGL), may result in increased or reduced eCB tone and signaling, consequently altering CB_1 function. In this regard, polymorphisms of the Cnr1 gene, which encodes the CB_1 receptor, may reduce or enhance G-protein- mediated signaling and have been associated to major depression, psychoses and schizophrenia (Martinez-Gras et al., 2006; Ponce et al., 2003). FAAH polymorphisms have been associated with drug abuse behaviours (Sipe et al., 2002) and ABDH12 mutations are associated to the onset of the neurodegenerative condition PHARC (Fiskerstrand et al., 2010).

CB_1 receptor signaling during embryonic development can be influenced as well by exposure to marijuana-derived cannabinoids, as our work and other's indicate, or by contact with drugs targeting indirectly the eCB system, like organophosphorus compounds (Nomura et al., 2008; Quistad et al., 2002). The neurobiological consequences of plant-derived cannabinoid intake on prenatal stages have been reviewed from the perspective of animal models and humans (Hurd et al., 2005; Jutras-Aswad et al., 2009; Schneider, 2009). According to the developmental stage in which CB_1 receptor signaling is affected, its interference may interfere with the development of different neural cell populations, including neuronal (present work, (Berghuis et al., 2007; Mulder et al., 2008; Watson et al., 2008; Wu et al., 2010) an glial (Aguado et al., 2006; Arevalo-Martin et al., 2007) cell generation and specification, and also the ulterior synaptic establishment and refinement (Keimpema et al., 2010; Li et al., 2009). Intriguingly, a recent study reports that the neuropsychiatric traits of THC exposure during embryonic development lead to an increased susceptibility to drug abuse in the subsequent generation (Szutorisz et al., 2014), the mechanisms of which are awaiting further explanation. Besides, cannabinoid intake during adolescence has been extensively studied, and many studies point to this fact as a risk factor for the onset of psychiatric conditions such as schizophrenia later on (Chadwick et al., 2013). From a wider point of view, clear evidence supports the occurrence of alterations in various components of the endocannabinoid system in psychoses (Saito et al., 2013; Zamberletti et al., 2012); what led to the cannabinoid hypothesis for schizophrenia (Emrich et al.,

1997; Muller-Vahl and Emrich, 2008). Also the glutamatergic hypothesis for schizophrenia (Paz et al., 2008) suggests that altered development of pyramidal neurons due to CB_1 malfunction could underlie the emergence of psychoses. However, it is still unknown whether some of the changes observed in elements of the endocannabinoid system contribute to the pathogenesis of the disease or, alternatively, their altered configuration reflect an attempt to adapt to aberrant neuronal homeostasis in order to counteract the changes of neuronal transmission (Eggan et al., 2008; Eggan et al., 2012). To note, polymorphisms of the CB_2 receptor-encoding gene, Cnr2, may associate with depressive syndromes and schizophrenia (Onaivi et al., 2008).

5. CONCLUSIONS

The main conclusions of this Thesis are listed following the same order as the results section.

- CB_1 cannabinoid receptors are present, albeit at low levels, in dorsal telencephalic progenitor cells.
- CB_1 signaling contributes to the identity and proliferation of cortical progenitor cells by modulating the activity of the transcription factor Pax6.
- Endocannabinoid signaling recruits the mTORC1 pathway to promote Pax6-dependent intermediate progenitor pool expansion.

- CB_1 receptors tune the gene expression regulatory network responsible for postmitotic pyramidal specification, by tempering the activity of the transcriptional repressor Satb2.
- Physiological endocannabinoid signaling is required for the appropriate generation and specification of corticospinal motor neurons (CSMN) and, therefore, skilled motor performance in the adulthood.
- Disrupted CB_1 receptor signaling elicited by embryonic Δ^9-tetrahydrocannabinol (THC) exposure impairs CSMN development and, as a consequence, negatively affects their function in the offspring.
- CNS development alterations ensued upon THC gestational exposure prone neuronal circuits to an epileptogenic configuration.

- CB_1 cannabinoid receptor signals radial migration in newborn pyramidal neurons. Its pro-migratory role rely specifically in the modulation of RhoA activity and, therefore, in the dynamic regulation of actin cytoskeleton.

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7. ADDENDA

Participation in research articles.

- **Addendum 1.** Palazuelos, J., Ortega, Z., Díaz-Alonso, J., Guzmán, M., Galve-Roperh, I. "CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling". J. Biol Chem. 287:1198-209 (2012)

- **Addendum 2.** Campos, AC., Palazuelos, J., Ortega, Z., Aguiar, D.C., Diaz-Alonso, J., Moreira, F.A., Guzmán, M., Guimarães, FS., Galve-Roperh, I. "The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system". Int J Neuropsychopharmacol. 9:1-13 (2013)

Participation in review articles.

- **Addendum 3.** Díaz-Alonso, J., Guzmán, M., Galve-Roperh, I. "Endocannabinoids via CB_1 receptors act as neurogenic niche cues during cortical development" Philos Trans R Soc Lond B Biol Sci. 367(1607): 3229-41 (2012)

- **Addendum 4.** Galve-Roperh, I., Chiurchiù, V., Díaz-Alonso, J., Bari, M., Guzmán, M., Maccarrone, M. "Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation" Prog. Lipid Res. 52(4): 633-50 (2013)

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CB_2 Cannabinoid Receptors Promote Neural Progenitor Cell Proliferation via mTORC1 Signaling*[S]

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Background: CB_2 cannabinoid receptors promote neural progenitor cell proliferation.
Results: CB_2 receptors induce neural progenitor cell proliferation and neurogenesis via activation of mTORC1 signaling.
Conclusion: CB_2 receptor/mTORC1-induced neural progenitor proliferation is relevant under physiological and pathological conditions such as cortical development and excitotoxicity-induced adult hippocampal neurogenesis.
Significance: Nonpsychotomimetic CB_2 receptor-selective ligands are promising molecules to manipulate neurogenesis.

The endocannabinoid system is known to regulate neural progenitor (NP) cell proliferation and neurogenesis. In particular, CB_2 cannabinoid receptors have been shown to promote NP proliferation. As CB_2 receptors are not expressed in differentiated neurons, CB_2-selective agonists are promising candidates to manipulate NP proliferation and indirectly neurogenesis by overcoming the undesired psychoactive effects of neuronal CB_1 cannabinoid receptor activation. Here, by using NP cells, brain organotypic cultures, and *in vivo* animal models, we investigated the signal transduction mechanism involved in CB_2 receptor-induced NP cell proliferation and neurogenesis. Exposure of hippocampal HiB5 NP cells to the CB_2 receptor-selective agonist HU-308 led to the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway, which, by inhibiting its downstream target p27Kip1, induced NP proliferation. Experiments conducted with the CB_2 receptor-selective antagonist SR144528, inhibitors of the PI3K/Akt/mTORC1 axis, and CB_2 receptor transient-transfection vector further supported that CB_2 receptors control NP cell proliferation via activation of mTORC1 signaling. Likewise, CB_2 receptor engagement induced cell proliferation in an mTORC1-dependent manner both in embryonic cortical slices and in adult hippocampal NPs. Thus, HU-308 increased ribosomal protein S6 phosphorylation and 5-bromo-2′-deoxyuridine incorporation in wild-type but not CB_2 receptor-deficient NPs of the mouse subgranular zone. Moreover, adult hippocampal NP proliferation induced by HU-308 and excitotoxicity was blocked by the mTORC1 inhibitor rapamycin. Altogether, these findings provide a mechanism of action and a rationale for the use of nonpsychotomimetic CB_2 receptor-selective ligands as a novel strategy for the control of NP cell proliferation and neurogenesis.

The endocannabinoids (eCBs)[5] 2-arachidonoylglycerol and anandamide are lipid signaling messengers involved in the homeostatic control of a large variety of functions of the nervous system (1). Thus, eCBs are produced on demand by activated postsynaptic cells and, by acting as retrograde messengers, control neurotransmitter release through presynaptic CB_1 cannabinoid receptors (2). CB_1 constitutes the most abundant neuronal G-protein-coupled receptor in some areas of the nervous system and is also involved in the control of neural cell proliferation/survival decision (3). CB_1 receptors exert a neuroprotective action, at least in part by controlling excessive glutamate release and excitotoxicity (4). In addition, they contribute to long term neuronal plasticity by promoting NP proliferation and excitotoxicity-induced neurogenesis (5–7). The other type of cannabinoid G protein-coupled receptor, the CB_2 cannabinoid receptor, is very abundant in some peripheral cells (*e.g.* lymphocytes and macrophages) and organs (*e.g.* spleen and thymus), and in the nervous system it is basically restricted to infiltrating immune cells and resident microglia/macrophages (8), oligodendrocyte progenitors (9), and neural progenitor/stem cells (NPs/NSC) (10). CB_2 receptors control the pro-inflammatory status of immune cells by modulating their Th1/Th2 phenotype, and this activity has important implications for neuronal survival under neuroinflammatory conditions occurring in animal models of neurodegenerative diseases, such as multiple sclerosis, Alzheimer disease, and Huntington disease, and upon acute ischemic brain injury (11). Because of the lack of undesired psychoactive effects of CB_2-selective ligands, therapeutic approaches aimed at targeting CB_2 receptors rather than CB_1 receptors are likely candidates to promote neuroprotection and neurorepair (12). CB_2 receptors

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[5] The abbreviations used are: eCB, endocannabinoid; KA, kainic acid; mTORC1, mammalian target of rapamycin complex 1; NSC, neural stem cell; NP, neural progenitor; p70S6K, 70-kDa ribosomal protein S6 kinase; S6, ribosomal protein S6; SGZ, subgranular zone; VZ/SVZ, ventricular/subventricular zone.

are present in embryonic stem cells (13) as well as in bone marrow-derived myeloid progenitors, in which they regulate cell proliferation and trafficking to the nervous system under neuroinflammatory conditions (14). In the nervous system, undifferentiated NSC/NPs also express functional CB_2 receptors (10, 15), but the final fate of CB_2-mediated newly born cell generation (10) is unknown; likewise, the signaling mechanism underlying CB_2 receptor actions remains to be elucidated.

CB_1 and CB_2 receptors are coupled to heterotrimeric G_i proteins, inhibition of adenylyl cyclase, and activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt (16). In addition, CB_1 receptors have recently been shown to modulate mammalian target of rapamycin complex 1 (mTORC1) signaling, which is in turn responsible for the cognitive impairment induced by Δ^9-tetrahydrocannabinol, the major active constituent of marijuana (17). mTORC1 is involved in the control of a plethora of cell functions by acting, for example, through the regulation of protein synthesis via phosphorylation of its downstream targets 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (18), which are essential elements in neuronal responses to synaptic activity and plasticity (19). In addition, mTORC1 is a major target of the PI3K/Akt pathway and thus also plays a central role in neural cell survival/death decision (18). For example, status epilepticus activates mTORC1, and this is required for the hippocampal alterations that contribute to the development of epilepsy, including mossy fiber sprouting, neuronal cell death, and neurogenesis (20). Considering this key position of mTORC1 in neural cell biology, as well as the involvement of the eCB system in finely tuning the balance between both excitatory and inhibitory neurotransmission (4, 21) and cell generation and death/survival (12, 22), here we investigated the signaling mechanism by which CB_2 receptors control NP cell proliferation and, in particular, the potential role of mTORC1 in this process. We show that CB_2 receptors present in NPs exert a proliferative effect that relies on the activation of the PI3K/Akt/mTORC1 axis and its downstream target p27Kip1. Furthermore, this CB_2 receptor-induced NP proliferation via mTORC1 is relevant in pathophysiological conditions such as NP proliferation during cortical development and excitotoxicity-induced adult hippocampal neurogenesis.

EXPERIMENTAL PROCEDURES

Materials—Founders of the CB_2 receptor knock-out mice colony were kindly donated by Nancy Buckley (National Institutes of Health, Bethesda) and were obtained by disrupting the CB_2 receptor gene by using homologous recombination in the embryonic stem cell line 129 (23). The CB_2 receptor-selective agonist HU-308 was kindly donated by Raphael Mechoulam (The Hebrew University, Jerusalem, Israel) and the HiB5 cells by Zaal Kokaia (Lund Stem Cell Center, Sweden). The antibodies employed in this study are detailed in supplemental Table 1.

Neural Progenitor Cultures—Multipotent self-renewing progenitors were obtained from embryonic E14.5 wild-type and nestin-GFP mice and grown as described previously (5) in chemically defined medium consisting of Dulbecco's modified Eagle's and F-12 media supplemented with N2 (Invitrogen), 0.6% glucose, nonessential amino acids, 50 mM Hepes, 2 μg/ml heparin-bound EGF, 20 ng/ml EGF, and 20 ng/ml FGF-2. Clonal neurospheres were derived from nonadherent dissociated cultures of NPs (1000 cells/ml), and experiments were carried out with early (up to 10) passage neurospheres. The HiB5 hippocampal progenitor cell line was grown as described (24) in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 10% (v/v) fetal calf serum. HiB5 cell cultures were incubated in 5% CO_2 at 33 °C, the proliferation-permissive temperature of the oncogenic tsA58 allele of the SV40 large T antigen. Incubation at 37 °C results in loss of proliferative capacity and neural differentiation. For Western blot and immunostaining analyses, HiB5 cells were pretreated with SR144528 (2 μM), LY-294,002 (5 μM), Akt inhibitor 1 (5 μM), rapamycin (50 nM), or PD98059 (10 μM) (10) for 30 min and subsequently treated with HU-308 for another 30 min. Stock solutions were prepared in dimethyl sulfoxide. No significant influence of dimethyl sulfoxide on any of the parameters determined was observed at the final concentration used (0.1% v/v). Control incubations included the corresponding vehicle content.

Proliferation and Cell Cycle Analyses—Neurosphere generation experiments were performed in 96-well dishes with 100 μl of medium. Neurospheres were pretreated with rapamycin (50 nM) for 30 min and then cultured in the continuous presence of HU-308 (50 nM) for 3 days. Subsequently the number of neurospheres per well was quantified. HiB5 cells were passaged, maintained, and analyzed at 33 °C. HiB5 cells were pretreated with SR144528 (2 μM) or rapamycin for 30 min, cultured in the continuous presence of HU-308 (50 nM) for 16 h, and with 5-bromo-2′-deoxyuridine (BrdU; 100 μg/ml) for 30 min followed by immunostaining. For cell cycle exit experiments, HiB5 cells were incubated with BrdU (100 μg/ml) for 30 min, treated with the indicated drugs for 48 h, and followed by immunostaining with rat monoclonal anti-BrdU and rabbit polyclonal anti-Ki-67 antibodies (supplemental Table 1). For flow cytometry analysis, HiB5 cells were trypsinized, permeabilized, and fixed in 1% (w/v) of bovine serum albumin and 30% ethanol/PBS and labeled with 5 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Fluorescence intensity was analyzed by using an LSR flow cytometer (BD Biosciences). Ten thousand cells per analysis were recorded.

Cell Transfection—HiB5 cells were transiently transfected 1 day after plating with 1 μg of pCMV6 mouse CB_2 receptor-expressing vector or empty vector (Origene, Rockville, MD) by using Lipofectamine 2000 following the instructions of the manufacturer (Invitrogen).

Organotypic Brain Cultures—Cortical brain slices were obtained from E14.5 mice and cultured under semidry conditions in neurobasal medium, B27 (1%), N2 (1%), glutamine (1%), penicillin/streptomycin (1%), fungizone (1%), and ciprofloxacine (5 μg/ml) as described previously (25). Brain slices were treated with the indicated drugs for 1 or 16 h and subsequently incubated with BrdU for 1 h. At the end of the experiment, brain slices were processed in 10-μm sections, and slice sections from equivalent regions of the rostral to caudal axis were analyzed by immunofluorescence.

RT-PCR—RNA was obtained with the RNeasy Protect kit (Qiagen, Valencia, CA) using the RNase-free DNase kit and cDNA synthesis kit (Roche Applied Science). Amplification of cDNA was performed with the specific primers indicated in supplemental Table 2. CB_2 receptor PCRs were performed using the following conditions: 1 min at 95 °C and 35 cycles (30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C). Finally, after a final extension step at 72 °C for 5 min, PCR products were separated on 1.5% agarose gels. The rest of the transcripts were detected as described previously (6).

Western Blot—Cleared cell extracts were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. After incubation with the correspondent primary antibodies (supplemental Table 1), blots were developed with the corresponding horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection kit. Loading controls were performed with an anti-α-tubulin antibody. Densitometric quantification of the luminograms was performed using a GS-700 imaging densitometer (Bio-Rad) and MultiAnalyst software (Bio-Rad).

Animal Procedures—Animal procedures were performed according to the European Union guidelines (86/609/EU) for the use of laboratory animals. Mice were housed (five per cage) with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12-h light/dark cycle. Adult CB_2 receptor knock-out mice (8 weeks old) and their respective wild-type littermates were obtained from heterozygote crosses (26). Mice were injected intraperitoneally with 100 mg/kg BrdU and vehicle (150 μl of PBS supplemented with 0.5 mg of defatted bovine serum albumin and 4% dimethyl sulfoxide) or 15 mg/kg HU-308, either alone or in combination with vehicle (150 μl PBS), or 6 mg/kg rapamycin (injected 30 min before vehicle/HU-308) daily for 5 days and perfused at either day 1 or 30 days later. For short term experiments, wild-type mice were administered a single intraperitoneal injection of 50 mg/kg BrdU and vehicle (150 μl PBS) or HU-308 (15 mg/kg) and perfused 3 h later. Kainate-induced excitotoxicity experiments were performed as described previously (6, 10). Animals were given a single injection of vehicle (150 μl PBS) or kainic acid (KA; 15 mg/kg) on the 1st day of treatment, alone or together with rapamycin, and sacrificed after 5 or 30 days.

Immunofluorescence and Confocal Microscopy—Fixed cell cultures, embryonic organotypic cortical sections (10 μm) attached to poly-L-lysine-coated slides, and adult coronal free-floating brain sections (30 μm) were processed as described (26). Briefly, after a 1-h blockade with PBS supplemented with 0.25% Triton X-100 and 10% goat serum, brain sections were incubated overnight at 4 °C with the indicated primary antibodies (supplemental Table 1), followed by incubation for 1 h at room temperature with secondary antibodies. The appropriate mouse, rat, and rabbit highly cross-adsorbed AlexaFluor 488, AlexaFluor 594, and AlexaFluor 647 secondary antibodies (Invitrogen) were used. Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024X1024 collection box. In slices, the number of $BrdU^+$, phospho-$p27Kip1^+$, and phospho-S6 highly immunoreactive positive cells present in the VZ/SVZ of the developing cortex was quantified, after image conversion to grayscale, in ImageJ by using the threshold tool and normalized to the total area selected for quantification. In adult mice, BrdU- and phospho-S6-positive cells were quantified in the SGZ of the hippocampus in a minimum of five coronal sections per animal. A 1-in-10 series of hippocampal sections located between 1.3 and 2.5 mm posterior to bregma were analyzed, and positive cells were normalized to the SGZ area determined with ×10 objective. The absolute number of positive cells was calculated considering the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them.

Data Analysis—Data are presented as means ± S.E. Significant differences between the groups were evaluated using an analysis of variance test followed by a Bonferroni post hoc comparison in the case of parametric population and Mann-Whitney test in the case of nonparametric populations. p values < 0.05 were considered significant.

RESULTS

Selective CB_2 Cannabinoid Receptor Activation Promotes Neural Progenitor Proliferation via PI3K/Akt/mTORC1 Signaling—NP cells have been shown to express CB_2 receptors in both neurosphere-derived progenitor cells and human/murine NSC lines (10, 15). To investigate the signal transduction mechanism of CB_2 receptors in NP cell proliferation, we first employed the HiB5 rat NP line (24). HiB5 cells cultured in proliferating conditions (33 °C) express CB_2 receptors, and after differentiation for 3 days at the restrictive temperature (37 °C), their expression was significantly reduced (supplemental Fig. 1). CB_1 receptors were also present in HiB5 cells but with an opposite pattern of expression, as CB_1 transcript levels increased with neural differentiation. In addition, transcripts of the putative eCB receptor GPR55 were also present in HiB5 cells, but their levels did not change with the differentiation status of the cells. CB_2 receptors are known to be coupled to PI3K/Akt and ERK activation (9, 10). Thus, we analyzed their functional coupling to downstream signaling in proliferating HiB5 cells by treatment with the CB_2-selective agonist HU-308 (27). HU-308 (50 nM) induced a rapid Akt activation as evidenced by Western blot analysis with anti-phospho-Ser-473-Akt antibody, and this effect was prevented by the CB_2-selective antagonist SR144528 (2 μM) (Fig. 1*A*). Likewise, HU-308 induced a time-dependent increase in the phosphorylation of downstream targets of PI3K/Akt signaling, including p70S6K and its substrate the ribosomal protein S6, as well as 4E-BP1 (Fig. 1*B*). Phosphorylation of p70S6K at Thr-389 indicated the involvement of mTORC1 activity in HU-308 signaling, in agreement with the observed phosphorylation of S6 at Ser-235/236 and 4E-BP1 at Ser-37/46 (18), thus indicating the involvement of mTORC1 in CB_2 receptor signaling. HU-308-induced phosphorylation of the mTORC1 downstream targets analyzed was prevented by co-incubation with SR144528 (Fig. 1*A*), therefore supporting the selectivity of HU-308 on CB_2 receptors. As S6 phosphorylation constitutes a well established readout of mTORC1 activity, we performed immunofluorescence analysis of phospho-S6 in HiB5 cells (Fig. 2*A*) and NPs derived from transgenic nestin-GFP mice (supplemental Fig. 2*A*).

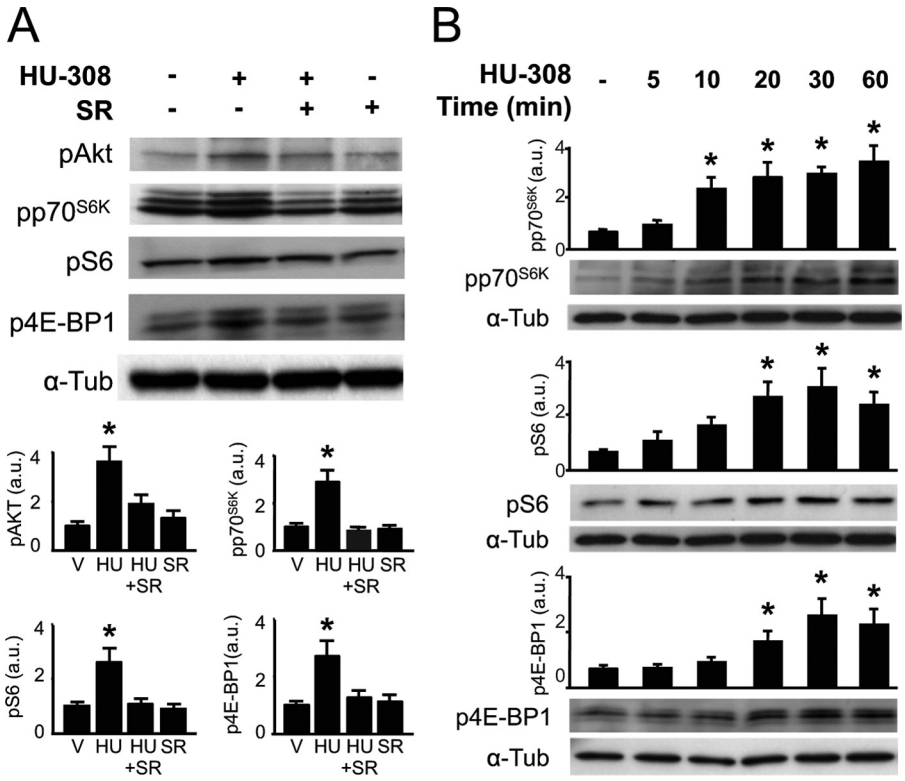

FIGURE 1. **CB_2 cannabinoid receptors signal through the PI3K/Akt/mTORC1 axis on HiB5 neural progenitor cells.** *A,* HiB5 cells were treated with HU-308 (50 nM) for 30 min alone or in the presence of SR144528 (*SR*, 2 μM). Western blot analysis was performed with anti-phospho-Akt, phospho-p70S6K, phospho-S6, and phospho-4E-BP-1 antibodies. Loading control was performed with anti-α-tubulin (*a-Tub*) antibody. Quantification of the relative phosphorylated protein and α-tubulin optical density is given in arbitrary units (*a.u.*). *B,* Western blot analysis was performed after HU-308 treatment for the indicated times with anti-phospho-p70S6K, phospho-S6, and phospho-4EBP-1 antibodies. Quantification of the relative phosphorylated protein was performed as above. Representative blots from four independent experiments are shown. *, $p < 0.05$ *versus* vehicle-treated cells.

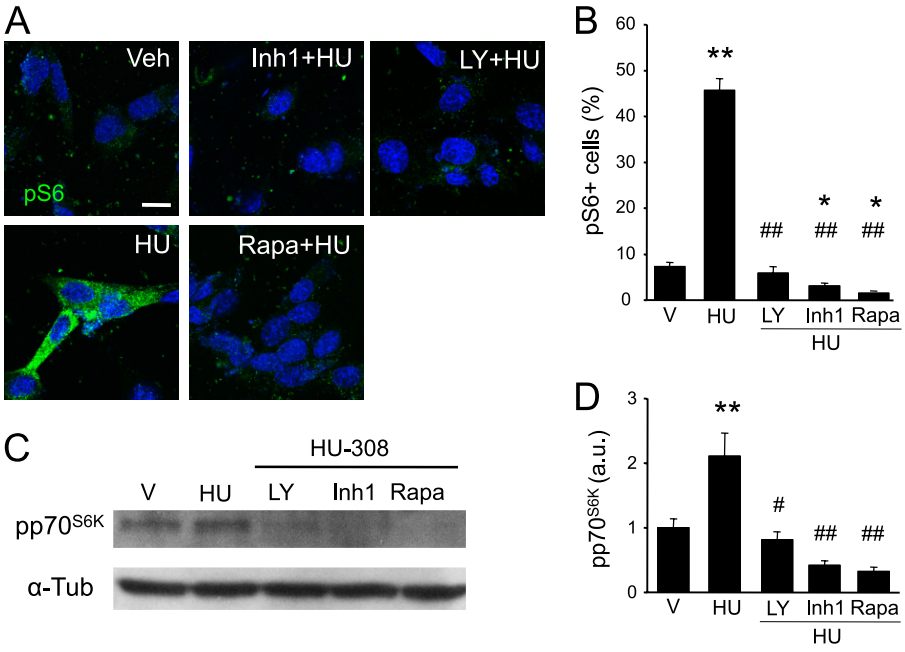

FIGURE 2. **CB_2 cannabinoid receptor activation of mTORC1 signaling depends on PI3K/Akt activation.** *A* and *B,* HiB5 cells were treated with HU-308 (*HU*; 50 nM) for 30 min in the absence or presence of LY-294,002 (*LY*; 5 μM), Akt inhibitor 1 (*Inh1*; 5 μM), and rapamycin (*Rapa*; 50 nM). The number of phospho-$S6^+$ HiB5 cells was quantified after incubation (as above) and immunofluorescence. Phospho-$S6^+$ cells were normalized to total cell number as identified by Hoechst 33342 counterstaining. Representative images are shown for each condition. *Veh,* vehicle. *Scale bar*, 10 μm. *C* and *D,* Western blot analysis of phospho-p70S6K were quantified and referred to loading control performed with anti-α-tubulin (*a-Tub*) antibody. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle (*V*)-treated cells; #, $p < 0.05$; ##, $p < 0.01$ *versus* HU-308-treated cells.

HU-308 administration increased phospho-S6-positive cells (Fig. 2, *A* and *B*). To investigate the mechanism involved in mTORC1 activation by HU-308, pharmacological inhibition studies were performed with the PI3K inhibitor LY-294,002, the Akt inhibitor 1, and the mTORC1 inhibitor rapamycin in HiB5 cells. The three compounds prevented HU-308-induced p70S6K and S6 phosphorylation (Fig. 2, *A–D*), further supporting the involvement of PI3K/Akt/mTORC1 in CB_2 receptor signaling. HU-308 alone or combined with the different inhibitors did not induce HiB5 cell death at the doses employed (supplemental Fig. 3). PI3K/Akt inhibitors and rapamycin reduced HU-308-induced p70S6K and S6 phosphorylation below basal levels in agreement with the involvement of this pro-survival signaling pathway in cortical development. The contribution of the ERK cascade to mTORC1 activation was analyzed with the MEK inhibitor PD98059. HU-308-induced ERK phosphorylation was prevented by PD98059, but this inhibitor failed to block HU-308-induced p70S6K phosphorylation and only exerted a marginal effect on HU-308-induced S6 phosphorylation (supplemental Fig. 4).

As CB_2 receptors present in NPs control cell proliferation (10, 15), we investigated by flow cytometry analysis of DNA content their signaling mechanism in the regulation of cell cycle progression in HiB5 cells. Thus, HU-308 reduced the fraction of cells in the G_0/G_1 compartment while increasing the fraction of cells in the S phase. This G_1-S phase progression was prevented by SR144528 (Fig. 3*A*) and rapamycin (Fig. 3, *B* and *C*), indicating the involvement of CB_2 receptors and mTORC1 in HU-308-induced cell cycle regulation. Likewise, SR144528 and rapamycin prevented HU-308-induced cell proliferation as determined by BrdU-positive cell quantification (supplemental Fig. 2*B*). In addition, pharmacological inhibition of PI3K/Akt with LY-294,002 and Akt inhibitor 1 blocked HU-308-induced HiB5 cell proliferation ($BrdU^+$ cells, vehicle, 25.94 ± 1.94; HU-308, 38.91 ± 3.24; LY-294,002+HU-308, 28.10 ± 2.16; I1+HU-308, 24.86 ± 1.08). The effect of HU-308 on cell proliferation was confirmed by using an NP-derived neurosphere formation assay. HU-308 administration increased neurosphere formation, and this pro-neurogenic action was prevented by mTORC1 inhibition (supplemental Fig. 2*C*). We further characterized CB_2 receptor-mediated regulation of cell cycle maintenance by analyzing BrdU-labeled cells (in S phase) and quantification of double-labeled cells with BrdU and Ki67, an endogenous marker of cycling cells, after incubation with HU-308. HU-308 promoted HiB5 cell cycle maintenance as reflected by the increased $BrdU^+Ki67^+$ cell fraction and the reduction of the $BrdU^+Ki67^-$ cell fraction (Fig. 4, *A* and *B*). In agreement with the flow cytometry studies, HU-308-induced cell cycle maintenance ($BrdU^+Ki67^+$ cells) was prevented by SR144528 and rapamycin (Fig. 4, *A–C*). HU-308 increased HiB5 cell number (Fig. 4*D*), and CB_2 receptor overexpression increased $BrdU^+$ cell number (Fig. 4*E*). Importantly, CB_2 overexpression induced an increase in HiB5 cell number that was blunted by rapamycin (Fig. 4*F*). Overall, these results demonstrate that CB_2 receptors in cultured NP cells evoke NP proliferation via PI3K/Akt/mTORC1 activation.

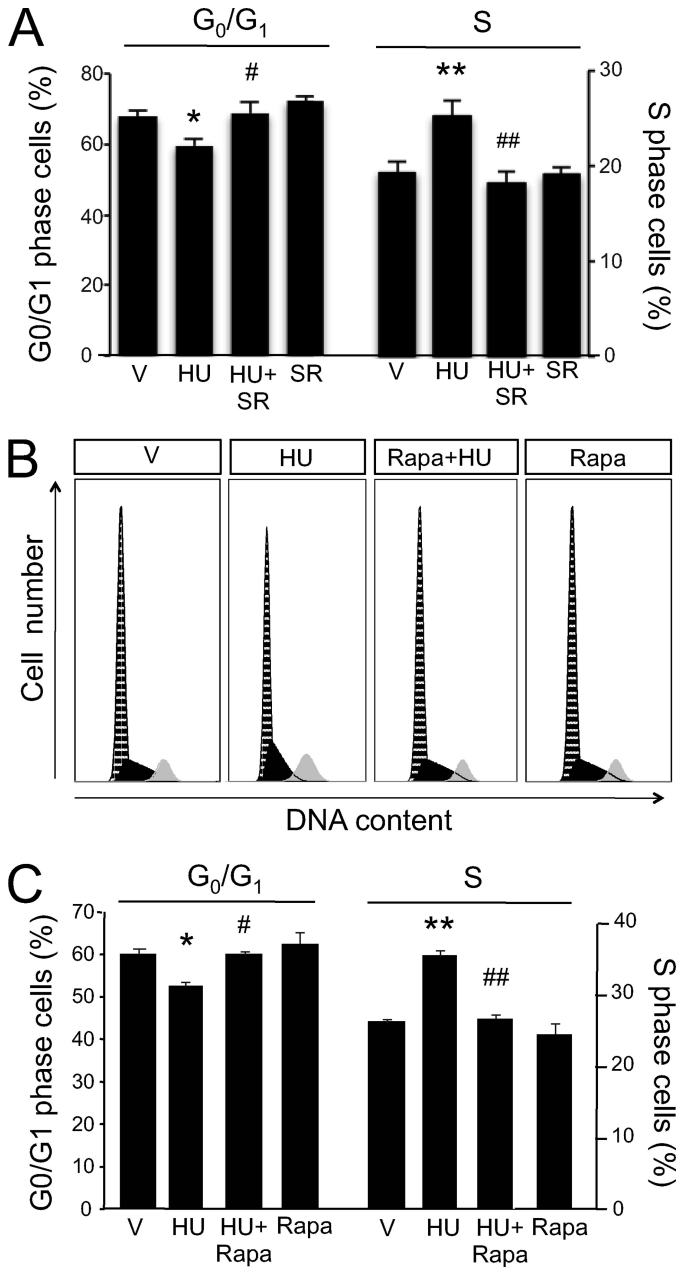

FIGURE 3. **CB_2 cannabinoid receptor activation promotes G_1/S phase progression of neural progenitors through activation of the PI3K/Akt/mTORC1 axis.** *A*, HiB5 cells were treated with HU-308 (*HU*; 50 nM) alone or in the presence of SR144528 (*SR*, 2 μM), and cell cycle analysis was performed after DNA content quantification by flow cytometry analysis. The relative fraction of cells in G_0/G_1 and S phase is shown. *B* and *C*, cell cycle analysis was performed after HU-308 administration alone or in the presence of rapamycin (*Rapa*; 50 nM). The fraction of cells in G_0/G_1 and S phase and a representative DNA histogram of each condition are shown. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle (*V*)-treated cells; #, $p < 0.05$; ##, $p < 0.01$ *versus* HU-308-treated cells.

CB_2 Cannabinoid Receptors Induce Cortical Progenitor Proliferation during Brain Development—To investigate the relevance of CB_2 receptor function in NSC proliferation in a more physiological environment, we employed organotypic mouse embryonic (E14.5) cortical slices. HU-308 induced NP cell proliferation as revealed by the increased BrdU-positive cell number in the VZ/SVZ, and this effect was prevented by SR144528 and rapamycin (Fig. 5, *A* and *B*). This increase in proliferation upon treatment with HU-308 was absent in CB_2 receptor

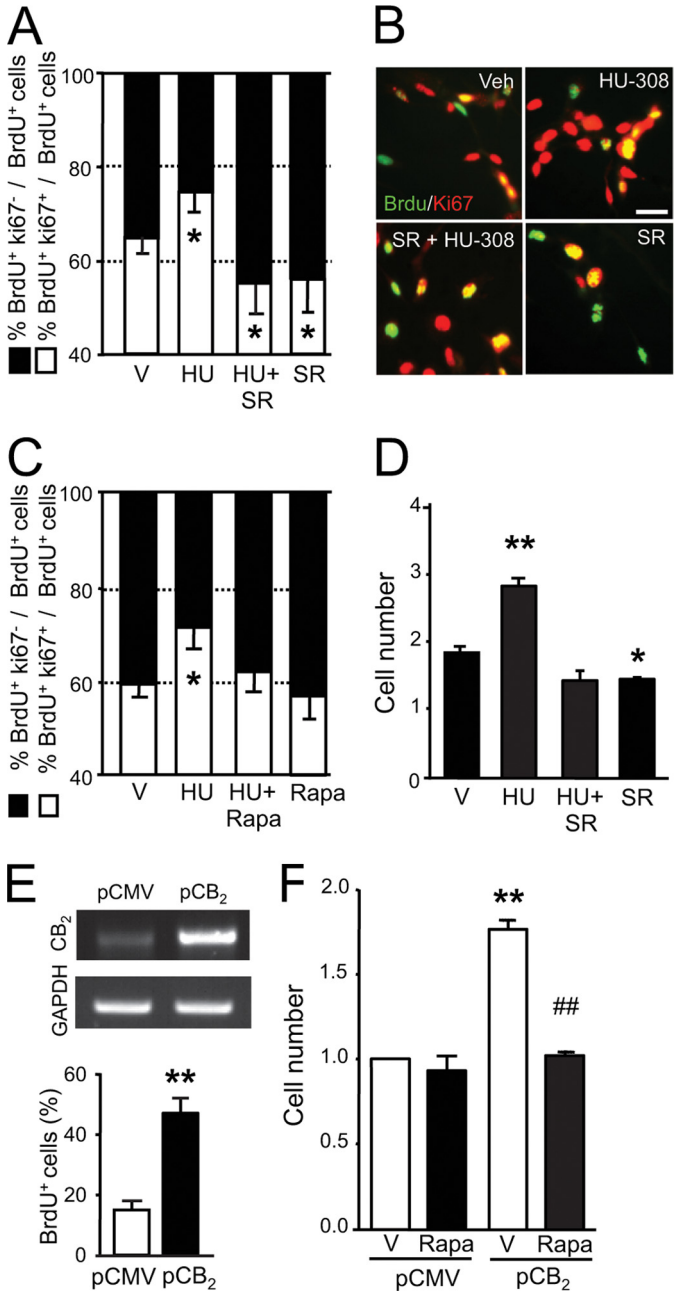

FIGURE 4. **CB_2 cannabinoid receptor activation promotes neural progenitor cell cycle maintenance through mTORC1 activation.** *A* and *B*, HiB5 cells were treated with HU-308 alone (*HU*; 50 nM) or in the presence of SR144528 (*SR*, 2 μM) for 48 h, and after immunofluorescence with anti-BrdU and anti-Ki67 antibodies (*green* and *red*, respectively), the percentage of $BrdU^+Ki67^+$ and $BrdU^+Ki67^-$ cells was quantified. Total cell number was determined by Hoechst 33342 counterstaining. Representative images of each condition are shown. *V*, vehicle. *Scale bar*, 20 μm. *C*, cell cycle maintenance of HiB5 cells was determined after HU-308 administration alone or in the presence of rapamycin (*Rapa*; 50 nM) as above. *D*, neurosphere-derived NPs were treated with HU-308 with or without SR144528 (2 μM) as above, and the number of cells was quantified in each condition. *E* and *F*, HiB5 cells were transiently transfected with pCMV6-mCB_2 or empty pCMV6 plasmids, and BrdU incorporation was quantified after 2 days (*lower panel*). Reverse transcription-PCR analysis of CB_2 receptor and GAPDH as loading control (*upper panel*) is shown. HiB5 cell number was quantified in CB_2 or control transfected cells after 2 days with and without rapamycin at 37 °C. Results correspond to four independent experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle-treated cells; ##, $p < 0.01$ *versus* HU-308-treated cells or vehicle-treated CB_2-transfected cells.

knock-out mice (data not shown). Moreover, short term HU-308 stimulation (1 h) induced Ser-235/236-S6 protein phosphorylation in undifferentiated VZ/SVZ cells (Fig. 5*C*, *arrows*) as well as in postmitotic neuroblasts that localize in the intermediate zone and developing cortical plate. The HU-308-induced increase in phospho-S6 immunoreactivity in the developing cortex was dependent on CB_2 receptors and mTORC1 as it was blocked by SR144528 and rapamycin (Fig. 5, *A–D*). These results support that the proliferative action of CB_2 receptor signaling in NPs is relevant during cortical development.

To investigate the mechanism by which CB_2 receptors/mTORC1 control progenitor cell cycle progression, we focused on the cyclin-dependent kinase inhibitor p27Kip1. This protein inhibits the G_1-S phase transition of NPs (28), regulates neuronal differentiation (29), and is a downstream target of mTORC1 (30). To test this possibility, E14.5 cortical slices were treated acutely with HU-308, and we quantified the number of phospho-Thr-157-p27Kip1-positive cells at the VZ/SVZ (Fig. 6, *A* and *B*). The HU-308-induced increase of phospho-p27 cell number was prevented by SR144528 and rapamycin. To investigate the mechanism of p27Kip1 regulation by CB_2 receptors, HiB5 cells were treated with HU-308 in the presence or absence of PI3K/Akt/mTORC1 inhibitors. HU-308 induced a time-dependent increase in p27Kip1 phosphorylation (Fig. 6*C*) that was prevented by LY-294,002, Akt inhibitor 1, and rapamycin (Fig. 6*D*). As p27Kip1 activity may be inhibited by mTORC1 via the downstream serum- and glucocorticoid-inducible kinase 1 (SGK1), thus allowing G_1-S progression (30), we analyzed the potential involvement of SGK1 as a molecular link between CB_2 receptor-induced PI3K/Akt/mTORC1 activation and p27Kip1 inhibition. HU-308 induced SKG_1 phosphorylation, which was prevented by rapamycin and the SGK1 inhibitor GSK-650394 (supplemental Fig. 5*A*). Furthermore, GSK-650394 prevented the HU-308-induced BrdU incorporation in HiB5 cells and the increase of neurosphere generation from NPs (supplemental Fig. 5, *B* and *C*). These results support that SGK1 may be responsible, at least in part, for the CB_2 receptor/mTORC1-mediated modulation of p27Kip1 phosphorylation and progenitor cell proliferation.

CB_2 Cannabinoid Receptors Promote Excitotoxicity-induced Hippocampal Progenitor Proliferation via mTORC1 Signaling—The aforementioned observations support that CB_2 receptors expressed by NPs are candidate targets for pharmacological manipulation to expand NP populations by inducing cell proliferation and, importantly, that CB_2 receptor action also occurs in the physiological NP niche of developing cortex slices. As both CB_2 receptors (10) and mTORC1 (20) are involved in hippocampal neurogenesis and the plasticity responses to excitotoxicity, we analyzed whether CB_2 receptors are coupled to mTORC1 signaling in the adult mouse hippocampus. HU-308 administration for 5 days increased NP proliferation as determined by quantification of $BrdU^+$ cells (Fig. 7*A*) (10). NP mobilization by HU-308 was also associated with an increased number of phospho-$S6^+$ cells, an effect that was absent in CB_2 receptor-deficient mice (Fig. 7*B*). Detailed confocal immunofluorescence analyses showed that HU-308-increased proliferation occurred in concert with S6 phosphorylation as nestin-labeled cells co-localized with phospho-S6 immunoreactivity

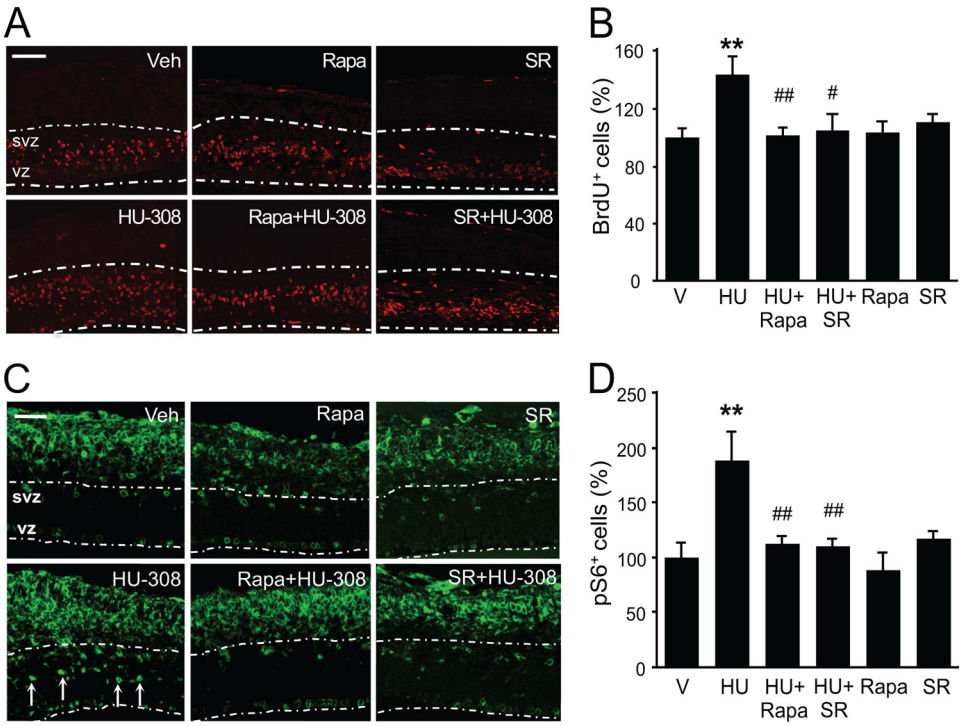

FIGURE 5. **CB_2 cannabinoid receptor agonist HU-308 induces cortical progenitor proliferation in organotypic cultures via mTORC1 signaling.** *A* and *B,* wild-type embryonic E14.5 cortical slices cultured in the presence of BrdU (10 μg/ml) were treated for 24 h with vehicle (*Veh*) or HU-308 alone (5 μM) or in the presence of SR144528 (*SR*; 25 μM) or rapamycin (*Rapa*; 250 nM). BrdU-positive cells in the ventricular and subventricular zone (*white dashed line*) were quantified and referred to the analyzed surface. *C* and *D,* phospho-S6-positive cells were quantified after immunofluorescence in slices treated for 1 h with vehicle or HU-308 alone or together with SR144528 or rapamycin. Results correspond to three independent experiments. *Scale bars, A* and *C,* 100 and 50 μm, respectively. **, $p < 0.01$ *versus* vehicle-treated slices; #, $p < 0.05$; ##, $p < 0.01$ *versus* HU-308-treated cells.

(Fig. 7*C*). After short term (3 h) administration, HU-308 selectively increased S6 phosphorylation in the subgranular zone (SGZ) (phospho-$S6^+$ cells, vehicle, 438 ± 30; HU-308, 1230 ± 240, $p < 0.01$) where NPs reside but not in differentiated neurons located in the granular zone (phospho-$S6^+$ cells, vehicle, 450 ± 54; HU-308, 522 ± 78, nonsignificantly different). Likewise, HU-308 stimulation (5 days) increased double-labeled $pS6^+BrdU^+$ newly born neurons (Fig. 7*D*). To determine the requirement of mTORC1 activation in HU-308-induced hippocampal NP proliferation, animals were co-administered with rapamycin, which blocked the $BrdU^+$ and phospho-$S6^+$ cell number increase induced by CB_2 receptor activation (Fig. 8, *A–D*).

We next analyzed the involvement of CB_2 receptors/mTORC1 signaling in excitotoxicity-induced hippocampal progenitor mobilization. CB_2 receptor ablation impaired excitotoxicity-induced NP mobilization as quantified 5 days after KA injection (Fig. 9*A*) (10). Likewise, immunofluorescence quantification revealed that KA induced an increase of phospho-$S6^+$ cells that did not occur in CB_2 receptor-deficient mice (Fig. 9*B*). In agreement, excitotoxicity-induced NP proliferation ($BrdU^+$ cells) and phospho-$S6^+$ cell number increase were prevented by rapamycin co-administration (Fig. 9, *C* and *D*). Finally, we investigated the long lasting outcome of excitotoxicity-induced progenitor mobilization by quantifying 30 days after KA injection the number of newly born $BrdU^+$ surviving cells together with the analysis of their expression of the mature neuronal marker NeuN (Fig. 9, *E* and *F*). Importantly, both the excitotoxicity-induced increase in $BrdU^+$ surviving cells and neurogenesis ($BrdU^+NeuN^+$ cells) were blunted in CB_2 receptor-deficient mice. These results reveal that CB_2 receptors, through mTORC1 signaling, mediate NP proliferation after excitotoxicity, thus resulting in hippocampal neurogenesis.

DISCUSSION

Here, we addressed the study of the signal transduction mechanism responsible for CB_2 cannabinoid receptor-mediated regulation of NP proliferation by means of pharmacological and gene expression manipulation in several experimental models of varying cellular complexity and physiological relevance. Using the hippocampal HiB5 progenitor cell line, NP-derived neurosphere cultures, organotypic embryonic cortical cultures, and hippocampal adult neurogenesis experiments, we show that CB_2 receptor activation promotes NP proliferation via PI3K/Akt/mTORC1 signaling both *in vitro* and *in vivo*. This proliferative effect of CB_2 receptors on NPs appears to be mediated by mTORC1-induced p27Kip1 inhibition via SGK1, which in turn allows cell cycle progression. Our results also support that the proliferative effect of CB_2 receptors in NPs is functional in neurogenic niches such as the developing cortex and the excitotoxicity-damaged adult hippocampal SGZ. CB_2 receptors are present and functional in diverse stem/progenitor cell lineages, including embryonic stem cells (13) and myeloid progenitors (14). Within neural cells CB_2 receptors are largely restricted to undifferentiated progenitor cells (10, 15) and, although still controversial, to discrete neuronal cell subpopulations (31). Biologically speaking, the notion that eCBs are signaling cues involved in the regulation of NSC/NP cell cycle

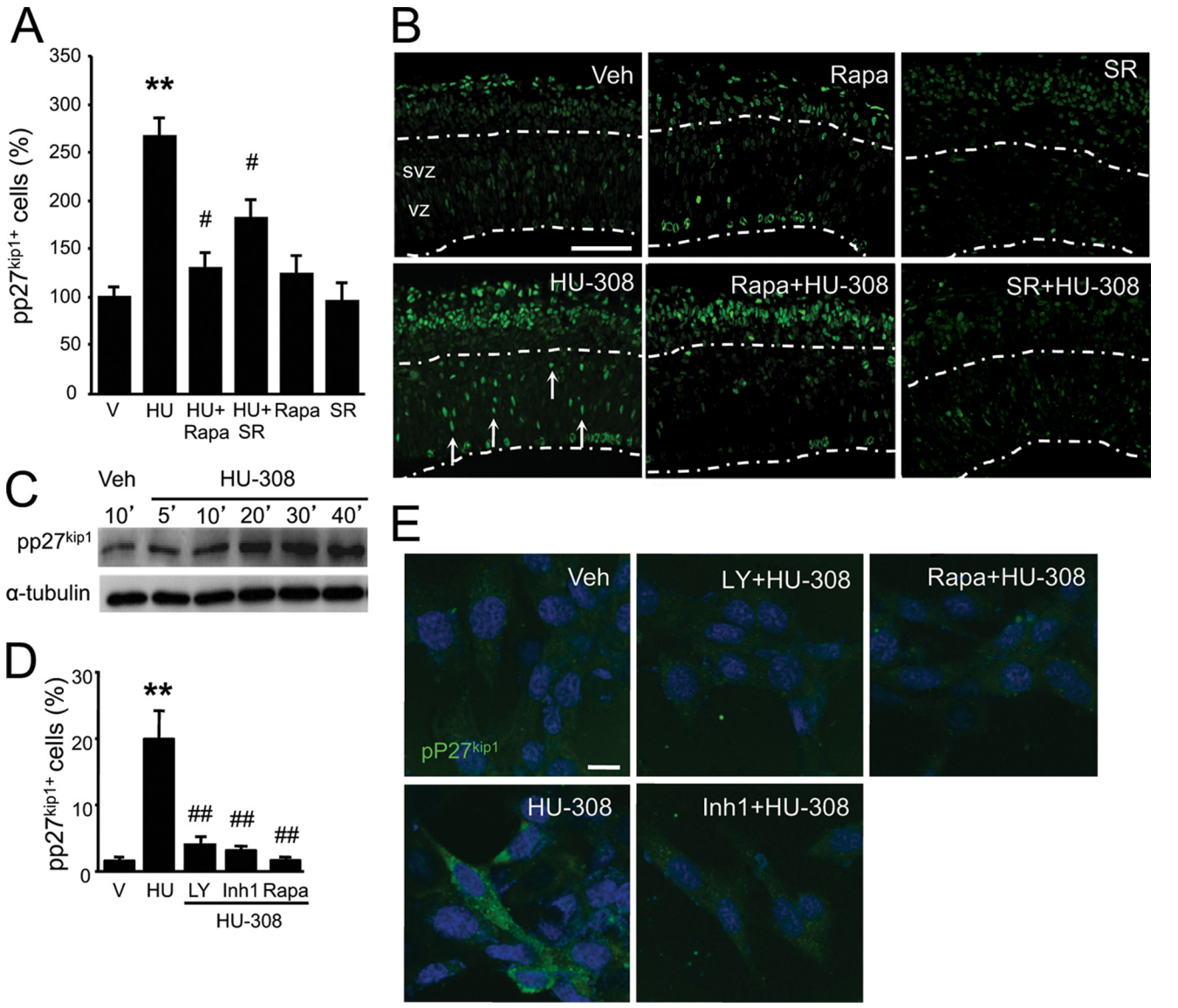

FIGURE 6. **CB_2 cannabinoid receptor-induced HiB5 cell proliferation is mediated by p27Kip1 inhibition.** *A* and *B*, wild-type embryonic E14.5 cortical slices were treated for 1 h with vehicle (*V*) or HU-308 alone (*HU*; 5 μM) or in the presence of SR144528 (*SR*; 25 μM) or rapamycin (*Rapa*; 250 nM). Phospho-p27Kip1$^+$ cells were quantified in the ventricular and subventricular zone (*VZ/SVZ*) after immunofluorescence and referred to the analyzed surface. Representative images are shown. *C*, HiB5 cells were treated with vehicle (*Veh*) or HU-308 (50 nM) for the indicated times, and p27Kip1 phosphorylation was determined by Western blot analysis. Loading control was performed with anti-α-tubulin antibody. *D* and *E*, HiB5 cells were treated for 30 min with vehicle or HU-308 (*HU*) alone or in the presence of LY294,002 (*LY*; 5 μM), Akt inhibitor 1 (*Inh1*; 5 μM), and rapamycin (*Rapa*; 50 nM). p27Kip1$^+$ cells were quantified by immunofluorescence in each condition. Representative images are shown. Results correspond to three independent experiments. *Scale bars, B* and *E*, 100 and 10 μm, respectively. **, $p <$ 0.01 *versus* vehicle-treated cells or slices; #, $p < 0.05$; ##, $p < 0.01$ *versus* HU-308-treated cells or slices.

progression and self-renewal is highlighted by the ability of cannabinoid receptors to contribute to the required proneurogenic niche that maintains NSC/NP cell populations during both brain development and adult neurogenesis (22, 32). On therapeutic grounds, the induction of progenitor cell proliferation and survival by CB_2 receptors adds to the proliferative and neuroprotective role of CB_1 receptors and opens new perspectives for the potential clinical utility of manipulating NP/stem cell mobilization with cannabinoid-based psychoactivity-devoid strategies (12).

CB_2 Cannabinoid Receptors and mTORC1 in Brain Development—During brain development, cortical progenitor proliferation and self-renewal are tightly coordinated with the onset of neural differentiation, cell cycle exit, and cell migration (33). CB_2 receptors present in undifferentiated neural cells promote mTORC1 signaling and downstream inhibition of the cell cycle inhibitor p27Kip1, which may play an important role in the balance of NP proliferation *versus* differentiation (28, 29). It is therefore conceivable that CB_2 receptor down-regulation along neuronal differentiation allows NPs to progress beyond self-renewal (*i.e.* via loss of mTORC1-mediated p27Kip1 inhibition) and to commit to migrate and differentiate. In this regard, CB_2 receptors have been recently shown to induce cell migration in explants of the postnatal SVZ enriched in progenitor cells, whereas the CB_2-selective agonist JWH-133 exerts a positive action on SVZ-derived neuroblast migration toward the olfactory bulb (34). Likewise, in the developing cortex, radial migration of neuroblasts is coordinated by the orienta-

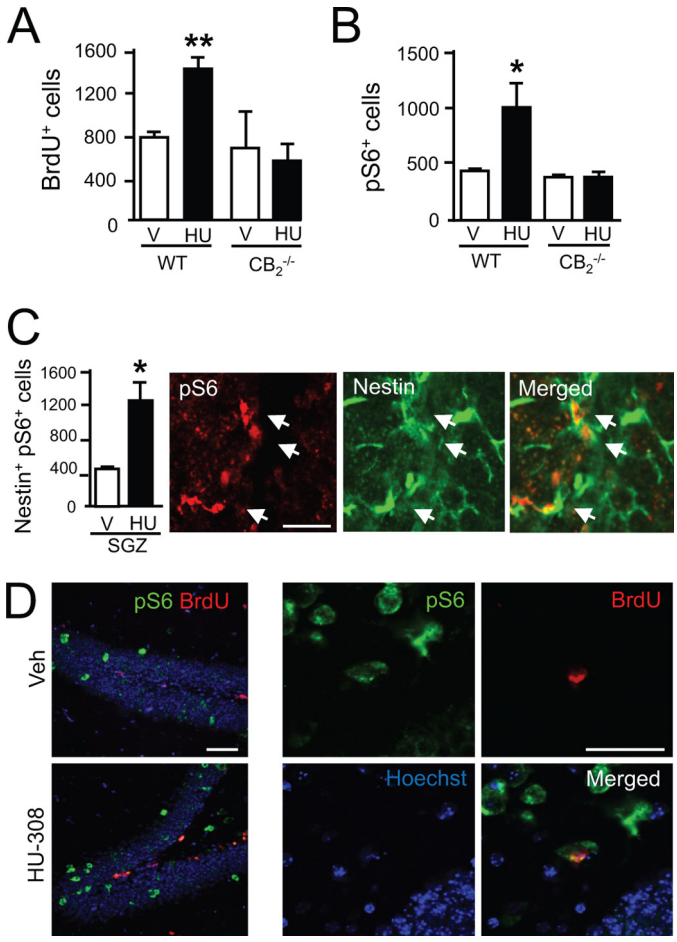

FIGURE 7. **HU-308-induced hippocampal progenitor proliferation is mediated by CB_2 receptors.** *A* and *B,* wild-type (*WT*) and CB_2 receptor-deficient mice were treated with vehicle (*V*; *white bars*) or HU-308 (*HU*; 15 mg/kg, daily intraperitoneal administration, *black bars*) for 5 days, and NP proliferation was quantified as $BrdU^+$ cell number. Immunoreactive phospho-$S6^+$ cells in the SGZ were also quantified ($n = 4$ in each group). *C,* WT mice were treated with vehicle or HU-308 for 3 h (*white* and *black bars*, respectively), and after immunofluorescence with selective antibodies for phospho-S6 and nestin, the number of double phospho-$S6^+$nestin$^+$ cells in the SGZ was quantified ($n = 4$ in each group). Representative high magnification image of HU-308-increased phospho-S6 immunoreactivity in nestin$^+$ cells is shown. *D,* representative images of wild-type mice treated for 5 days with vehicle or HU-308 indicating BrdU and phospho-S6 co-localization. *Scale bar,* 50 μm. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle-treated mice.

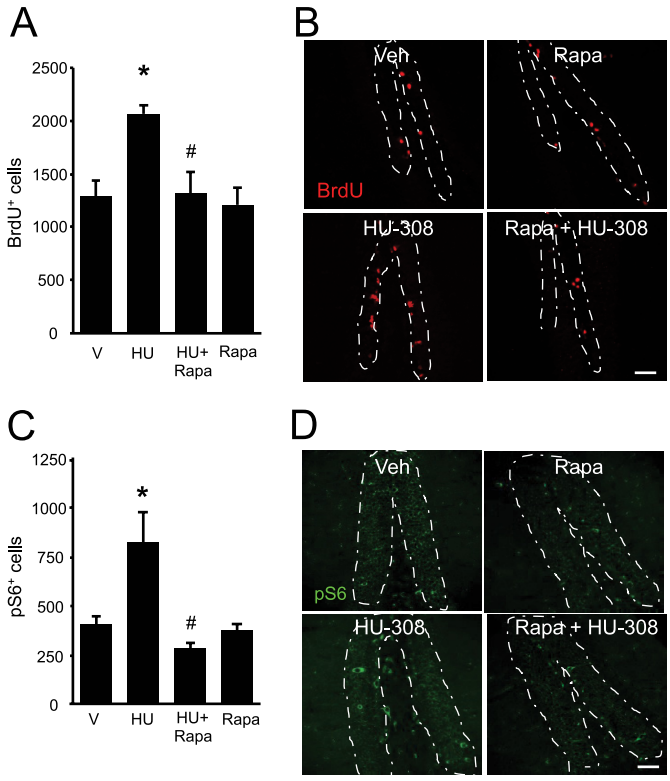

FIGURE 8. **HU-308-induced hippocampal progenitor proliferation is mediated by mTORC1 signaling.** *A* and *B,* wild-type (*WT*) mice were treated with vehicle (*V* or *Veh*) or HU-308 (*HU*; 15 mg/kg, daily intraperitoneal administration) for 5 days alone or in the presence of rapamycin (*Rapa*; 6 mg/kg), and NP proliferation was quantified as $BrdU^+$ cell number ($n = 6$ in each group). Representative immunofluorescence images are shown. *C* and *D,* immunoreactive phospho-S6 cells in the SGZ in the same treated animals were also quantified. *Scale bars,* 50 μm. *, $p < 0.05$; *versus* vehicle-treated mice; #, $p < 0.05$ *versus* HU-308-treated mice.

tion of the cell division plane with respect to the ventricular surface, which controls symmetric and asymmetric divisions. Thus, the apical polarity complex regulates the VZ/SVZ progenitor pool size by coordinating cell proliferation and differentiation through mTORC1 activity via the Pals1 protein (35). Maintenance of the balance between self-renewal and survival is associated with Pals1/mTORC1 activity and p27Kip1 phosphorylation. p27Kip1 exerts a dual role and, beyond cell cycle regulation, promotes neuronal differentiation and migration through distinct and separable mechanisms (29). CB_2 receptor-mediated mTORC1 activation and downstream p27Kip1 inhibition may therefore constitute part of the molecular switch that coordinates cell proliferation and migration of VZ/SVZ cortical progenitors.

The signaling coupling of cannabinoid receptors to downstream effectors, and in particular to mTORC1, seems to be highly dependent on the pathophysiological cell context. On the one hand, CB_1 receptors activate mTORC1 in hippocampal neurons, which, by regulating protein synthesis, is responsible for Δ^9-tetrahydrocannabinol-induced cognitive impairment (17). On the other hand, CB_1 receptors exert a proapoptotic action on transformed glial cells via Akt/mTORC1 inhibition (36). In addition, oligodendrocyte progenitor survival and differentiation is regulated by CB_1 receptors via PI3K/Akt signaling (9) and cannabinoid agonists induce myelin basic protein expression and myelination during postnatal development (37) in a process that is blocked *in vitro* by rapamycin (38). This suggests a role of CB_1 receptor-mediated PI3K/Akt/mTORC1 signaling in oligodendroglial cells, which is in agreement with the role of Rheb1-mediated activation of mTORC1 in myelination and oligodendrocyte differentiation (39). Importantly, the tuberous sclerosis complex proteins hamartin and tuberin function as upstream regulators of Rheb1 and therefore of mTORC1, and mutations of those proteins contribute to cortical dysplasia and intractable epilepsy (40). Hence, the role of CB_2 receptor/mTORC1 signaling during cortical development may have important implications regarding the potential involvement of CB_2 receptor misexpression in the development of tuberous sclerosis complex disorder (41).

CB_2 Cannabinoid Receptors and mTORC1 in Epileptogenesis—The vast majority of the studies regarding the role of the endo-

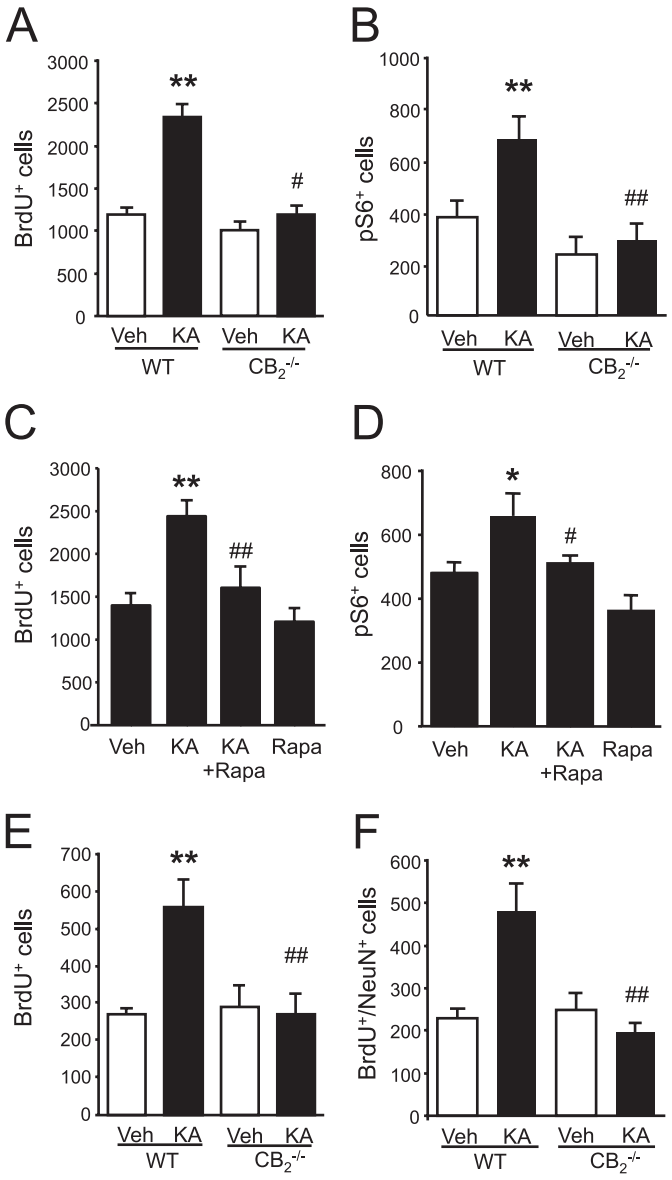

FIGURE 9. **Excitotoxicity-induced hippocampal progenitor proliferation is mediated by CB_2 cannabinoid receptor/mTORC1 signaling.** *A* and *B,* wild-type (*WT*) and CB_2 receptor-deficient mice were treated with vehicle (*Veh*; *white bars*) or kainic acid (*KA*, 15 mg/kg, *black bars*), and NP proliferation was determined by quantifying $BrdU^+$ cells 5 days after injury. Immunoreactive phospho-S6 cells in the same animals were also quantified ($n = 4$ in each group). *C* and *D,* WT mice were treated with vehicle or KA (15 mg/kg) with or without rapamycin (*Rapa*; 6 mg/kg), and NP proliferation was determined by quantifying $BrdU^+$ cells 5 days after injury. Immunoreactive phospho-S6 cells in the same animals were also quantified ($n = 6$ of each group). *E* and *F,* WT and CB_2 receptor-deficient mice treated with vehicle or KA as described above and analyzed 30 days after injury. The number of $BrdU^+$ cells and double $BrdU^+NeuN^+$ cells were quantified ($n = 7$ in each group). *Scale bar,* 50 μm. *, $p < 0.05$; **, $p < 0.01$ *versus* vehicle-treated mice; #, $p < 0.05$; ##, $p < 0.01$ *versus* HU-308-treated mice.

cannabinoid system in epileptogenesis has focused on the contribution of CB_1 receptors (1) and, in particular, on the protective role of presynaptic CB_1 receptor activation upon on-demand eCB synthesis as a consequence of excessive neuronal activity (4). Excitatory neuronal activity induced by KA administration induces seizures and a series of subsequent long term neuronal adaptive responses leading to epileptogenesis. Hippocampal progenitors respond to excitotoxicity with increased proliferation and neurogenesis, which may contribute to palliate neuronal cell loss or, on the contrary, participate in the generation of aberrant processes that contribute to the development of epilepsy (*e.g.* synaptic remodeling and axonal sprouting) (42). mTORC1 signaling is known to impact excitotoxicity-induced neuronal remodeling and follows a biphasic kinetic pattern with a rapid activation phase within hours and a subsequent sustained period that lasts several days (20, 43). Thus, mTORC1 inhibition prior to KA administration blocks acute and sustained seizure-induced mTORC1 activation, whereas late rapamycin administration fails to inhibit excitotoxicity-induced neurogenesis (20). This is in agreement with the dual contribution of mTORC1 signaling to cell proliferation (20, 44) and neuronal differentiation and migration (43, 45). The involvement of CB_2 receptors in excitotoxicity-induced NP proliferation (10) via PI3K/Akt/mTORC1 activation (this study) suggests that CB_2 receptor antagonists might be candidates to prevent seizure-induced neurogenesis, therefore attenuating the development of epileptogenesis.

CB_2 receptors exert a prominent role in the regulation of microglial activation and neuroinflammation (8), and we found that in $CB_2^{-/-}$ mice there is a complete absence of hippocampal NP proliferation induced by excitotoxicity (this study and see Ref. 10). This finding suggests that CB_2 receptors, aside from actively promoting progenitor proliferation in a cell-autonomous manner, may be responsible for injury-induced microglial priming and for the release of neurogenesis-inducing factors (46). The role of the eCB system in epileptogenesis and long term neural plasticity is relevant not only in animal models but perhaps also in the human epileptic brain (1). Thus, CB_1 receptors are regulated in a dynamic manner by synaptic activity and become down-regulated in the human epileptic hippocampus (47, 48). However, the possible involvement of CB_2 receptors in these pathological events is still unknown. In opposition to the aforementioned potential benefit of blocking CB_2 receptors expressed in the NP cell compartment to palliate epileptogenesis, the anti-inflammatory role of CB_2 receptors in microglial and nervous system-infiltrating immune cells could contribute to attenuate neural cell loss (14, 26) and epileptogenesis (49). Altogether, these observations suggest that the presence of functional CB_2 receptors in neurogenic niches *in vivo* might open new perspectives aimed at palliating the pathological consequences of aberrant neurogenesis, particularly in epileptogenesis.

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ARTICLE

The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system

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Abstract

Cannabidiol (CBD), the main non-psychotomimetic component of the plant *Cannabis sativa*, exerts therapeutically promising effects on human mental health such as inhibition of psychosis, anxiety and depression. However, the mechanistic bases of CBD action are unclear. Here we investigate the potential involvement of hippocampal neurogenesis in the anxiolytic effect of CBD in mice subjected to 14 d chronic unpredictable stress (CUS). Repeated administration of CBD (30 mg/kg i.p., 2 h after each daily stressor) increased hippocampal progenitor proliferation and neurogenesis in wild-type mice. Ganciclovir administration to GFAP-thymidine kinase (GFAP-TK) transgenic mice, which express thymidine kinase in adult neural progenitor cells, abrogated CBD-induced hippocampal neurogenesis. CBD administration prevented the anxiogenic effect of CUS in wild type but not in GFAP-TK mice as evidenced in the novelty suppressed feeding test and the elevated plus maze. This anxiolytic effect of CBD involved the participation of the CB_1 cannabinoid receptor, as CBD administration increased hippocampal anandamide levels and administration of the CB_1–selective antagonist AM251 prevented CBD actions. Studies conducted with hippocampal progenitor cells in culture showed that CBD promotes progenitor proliferation and cell cycle progression and mimics the proliferative effect of CB_1 and CB_2 cannabinoid receptor activation. Moreover, antagonists of these two receptors or endocannabinoid depletion by fatty acid amide hydrolase overexpression prevented CBD-induced cell proliferation. These findings support that the anxiolytic effect of chronic CBD administration in stressed mice depends on its proneurogenic action in the adult hippocampus by facilitating endocannabinoid-mediated signalling.

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Key words: Anxiety, endocannabinoid system, hippocampal neural progenitor, neurogenesis, stress.

Introduction

Cannabidiol (CBD), the main non-psychotomimetic cannabinoid derived from the plant *Cannabis sativa*, possesses a wide therapeutic potential (Izzo et al., 2009). In the context of psychiatric disorders, CBD administration has been shown to exert antipsychotic and anxiolytic effects in humans (Bergamaschi et al., 2011; Leweke et al., 2012) as well as in several animal models (Guimaraes et al., 1990; Zuardi et al., 1995; Casarotto et al., 2010). However, the study of CBD actions has been mostly restricted to its acute effect, whereas its efficacy after chronic administration is largely unknown. Moreover, despite the growing interest in this compound, its mechanism of action is still unclear and numerous receptors have been proposed to mediate its different responses (Izzo et al., 2009). Thus, for example, CBD may regulate, directly or indirectly, the activity of peroxisome proliferator-activated receptor γ (PPARγ), serotonin 5-HT_{1A} receptor, adenosine transporter, some members of the TRPV family and metabotropic CB_1 and

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CB_2 cannabinoid receptors. CB_1 and CB_2 receptors constitute the main molecular targets of psychotomimetic plant-derived cannabinoids such as $\triangle^9$-tetrahydrocannabinol (THC) and they are physiologically engaged by endogenous ligands, namely, the endocannabinoids (eCBs) 2-arachidonoylglycerol and anandamide (Heifets and Castillo, 2009). Although CBD administration has been found to exert some of its actions independently of CB_1/CB_2 receptors (Pertwee et al., 2010), in other studies CBD has been reported to regulate these receptors (Thomas et al., 2007; Bitencourt et al., 2008; Casarotto et al., 2010; Castillo et al., 2010) or to mimic eCB actions by impairing anandamide hydrolysis or re-uptake, thus facilitating eCB-mediated neuromodulation (Bisogno et al., 2001). In agreement, the antipsychotic actions of CBD have been proposed to be mediated by increased anandamide levels (Leweke et al., 2012).

Increasing evidence shows that adult hippocampal neurogenesis is associated with the regulation of cognitive and emotional functions and impaired neurogenesis has been implicated in psychiatric disorders such as anxiety and depression (David et al., 2010). Adult hippocampal neurogenesis is required to buffer stress and endocrine responses (Snyder et al., 2011) and attenuation of hippocampal neurogenesis promotes anxiety-related behaviours (Revest et al., 2009). Thus, factors that decrease neurogenesis impair learning and certain forms of memory and facilitate the appearance of stress-related disorders (David et al., 2010; Deng et al., 2010). On the other hand, proneurogenic stimuli such as enriched environment, running, social interaction and some antidepressant drugs exert antidepressive and anxiolytic actions (van Praag et al., 1999; Santarelli et al., 2003; Schloesser et al., 2010). Blockade of adult neurogenesis prevents some of the behavioural effects of antidepressants (Santarelli et al., 2003; David et al., 2009), although increased neurogenesis alone is not sufficient to reproduce the behavioural actions of antidepressant or anxiolytic drugs (Sahay et al., 2011). Altogether, these findings have led to the proposal that promoting adult neurogenesis may be a novel therapeutic strategy to palliate anxiety and mood disorders (Surget et al., 2011). In recent years the eCB system has been implicated in the regulation of adult neurogenesis (Galve-Roperh et al., 2009). Thus, CB_1 receptors enhance basal and excitotoxicity-induced hippocampal neural progenitor cell proliferation (Aguado et al., 2005, 2007) and chronic CB_1 receptor activation exerts a proliferative and proneurogenic action linked to anxiolytic and antidepressant-like effects (Jiang et al., 2005). In addition, CB_2 receptors also promote neural progenitor proliferation (Palazuelos et al., 2006; Goncalves et al., 2008), although the consequences of this CB_2 receptor-evoked progenitor expansion in neurogenesis and the regulation of depression and anxiety are as yet unknown.

On the basis of this background, the present work was undertaken to investigate: (i) the potential therapeutic effect of chronic CBD administration in anxiety; (ii) whether this behavioural effect of CBD relies on hippocampal neurogenesis; (iii) the molecular mechanism of CBD anxiolytic action.

Method and materials

Materials

The following materials were kindly provided: CBD by THC-PHARM (Germany); SR141716 and SR144528 by Sanofi Aventis (France); the HiB5 cell line by Z. Kokaia (Lund Stem Cell Center, Sweden).

Animal procedures

Animal procedures were performed according to the European Union (86/609/EU) and Brazilian guidelines for the use of laboratory animals. Mice (3 months old) were housed (five per cage) with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12 h light/12 h dark cycle (lights on 07:00 hours). Procedures were designed to minimize the number of animals used and their suffering. In order to evaluate the effect of chronic CBD treatment in neurogenesis the groups received, at the beginning of the chronic unpredictable stress (CUS) paradigm, daily i.p. injections of 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg) during 3 consecutive days. Hemizygous male mice expressing thymidine kinase under the control of the glial fibrillary acidic protein (GFAP) promoter (code B6.Cg-Tg (GFAPTk) 7.1Mvs/J), as well as their corresponding wild-type (WT-C57BL/6J) littermates, were purchased from The Jackson Laboratory (USA). Experiments of depletion of astroglial neural progenitors and hippocampal proliferation were performed in GFAP-TK mice as previously described (Palazuelos et al., 2009). Ganciclovir (GCV; 100 mg/kg, Roche Farma, Spain) was administered by i.p. injection daily (7 d) starting 5 d before the beginning of the chronic stress procedures. GFAP-TK transgenic and WT mice, subjected (or not) to the CUS paradigm for 14 d, received daily i.p injections of CBD (30 mg/kg) or vehicle (150 μl PBS supplemented with 0.5 mg defatted bovine serum albumin and 4% dimethylsulfoxide) 2 h after the daily stressor. The CBD dose used was the same that induced acute antidepressive-like effects in mice (Zanelati et al., 2010). On days 14 and 15 the mice were subjected to the elevated plus-maze (EPM) and novelty suppressed feeding (NSF), respectively, to evaluate anxiety-like behaviours. The EPM and NSF tests were performed just before and 24 h after the last CBD injection, respectively. This 24-h interval between drug administration and test performance is sufficient, according to the pharmacokinetics of CBD in mice, to ensure complete drug elimination before the behavioural test (Deiana et al., 2012). In some experiments AM251 (1.0 mg/kg, Tocris- Bristol, UK) was administered i.p. 10 min prior to CBD (30 mg/kg) injection

(Casarotto et al., 2010). Mouse brains were perfused after behavioural analyses and processed for immunofluorescence analysis.

Chronic unpredictable stress

WT and GFAP-TK mice were subjected, during the light period of the cycle, to a variant of the chronic mild stress paradigm (Santarelli et al., 2003) for 14 d. Different mild stressors were used randomly: bedding alterations (sawdust removal, substitution of sawdust by 5-mm deep water for 4 h); 2 h restraint stress session; 10 min forced swimming; reversal of light/dark cycle; four light/dark successive alterations in 24 h (30 min of duration).

NSF test

The NSF behaviour test was performed in a 5 min test session as previously described (Santarelli et al., 2003). The apparatus consisted of an acrylic box ($40 \times 40 \times 30$ cm) with the floor covered by 2 cm of sawdust. Twenty-four hours before the test, all animals were food deprived. On the day of the test a single regular chow pellet was placed in a white platform located in the middle of the box. Each animal was placed in one of the apparatus corners and the latency to start to eat in the new environment was measured. The stopwatch was immediately stopped when the mouse bit the chow, using its forepaws sitting on its haunches. After the test all animals were returned to their home cages and the amount of food consumed in 5 min was measured. Basal feeding latency of control mice differed in separate CUS experiments (Figs. 3, 4). As these experiments were performed in different laboratories (Brazil and Spain), it is conceivable that differences in animal housing, environment and handling conditions are responsible for this.

EPM test

The EPM apparatus composed two open arms ($30 \times 7 \times 0.25$ cm), as opposed to two enclosed arms ($30 \times 7 \times 15$ cm), was elevated 60 cm from the floor and was made of dark grey plastic. At the beginning of the test, each mouse was placed in the central area of the apparatus with its head facing an enclosed arm. The test duration was 5 min and was performed in a sound attenuated and temperature-controlled (25 ± 1 °C) room, illuminated by three 40-W fluorescent bulbs placed 4 m above the apparatus. The Anymaze software (Stoelting Co., USA) was employed for behavioural analysis. It detects the position of the animal in the maze and calculates the number of entries and time spent in open and enclosed arms. Enclosed-arm entries were considered as an indicator of locomotor activity, whereas percentage of time spent in open arms and percentage open-arm entries were used as measures of anxiety. In the experiment performed with GFAP-TK mice (Fig. 3) only data from stressed mice were analysed due to a recording problem during the EPM procedure.

eCB quantification

Tissue samples, stored at −80 °C until the moment of analysis, were weighed and homogenized in an ice-cold glass dounce-homogenizer in a mixture 2:1:1 (v:v:v) of chloroform:methanol:Tris HCl 50 mM (pH 7.5). The organic and aqueous layers were separated by centrifugation (4500 *g*, 2 min) and the organic layer transferred to a clean vial and dried under a stream of argon. This fraction was reconstituted in 50 μl acetonitrile and analysed by high-pressure liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS analysis was performed using an Agilent 1200LC-MSD VL instrument (Agilent Technologies, USA). LC separation was achieved with a Zorbax Eclipse Plus C18 column (5, 4.6×50 mm; Agilent Technologies) together with a guard column (5, 4.6×12.5 mm). The gradient elution mobile phases consisted of A (95:5 water:acetonitrile) and B (95:5 acetonitrile:water), with 0.1% formic acid as the solvent modifier. The gradient (flow rate of 0.5 ml/min) started at 0% B (for 5 min), increased linearly to 100% B over the course of 45 min and decreased to 0% B for 10 min before equilibrating for 5 min with an isocratic gradient of 0% B. MS analysis was performed with an electrospray ionization source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set at 70 V. The drying gas temperature was 350 °C, the drying gas flow rate was 10 l/min and the nebulizer pressure was 20 × psi. LC-MS measurements were made by selected ion monitoring in positive mode. Fractions were quantified by measuring the area under the peak and normalized using d8-AEA, d8-2-AG or d5-PEA (Cayman Chemical Company, USA) as internal standards. Absolute AEA, 2-AG and palmitoylethanolamide (PEA) levels were estimated by comparison with their respective deuterated standards. eCB levels were referred to tissue weight.

Microscopy

Adult coronal free floating brain sections ($30 \times \mu$M) or fixed cell cultures were processed as described (Palazuelos et al., 2009). Briefly, after 1 h blockade with PBS supplemented with 0.25% Triton X-100 and 5% goat serum, brain sections were incubated overnight at 4 °C with the rat monoclonal anti-BrdU (Abcam, UK) or rabbit polyclonal anti-doublecortin (Santa Cruz Biotechnology, USA) primary antibodies, followed by incubation for 1 h at room temperature with the appropriate highly cross-adsorbed secondary antibodies (Invitrogen, USA). Doublecortin immunoreactivity was detected by the avidin–biotin immunoperoxidase method (Vectastain ABC kit; Vector Lab, USA) and the product of the reaction was revealed by adding the chromogen 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical, USA). Confocal fluorescence images were acquired by using Leica TCS-SP2 software (Wetzlar, Germany) and SP2 microscope with two passes by Kalman filter and a 1024×1024 collection box. *In vivo*, BrdU- and doublecortin-positive

cells were quantified in the subgranular zone of the hippocampus in a minimum of five coronal sections per animal. A 1-in-10 series of hippocampal sections located between 1.3 and 2.5 mm posterior to bregma were analysed and positive cells were normalized to the dentate gyrus area determined with X10 objective. The absolute number of positive cells was calculated considering the total hippocampal volume as determined by the sum of the areas of the sampled sections multiplied by the distances between them. Doublecortin immunoreactivity was quantified using a computerized image analysis system (ImagePro software).

Neural progenitor cultures

The HiB5 hippocampal progenitor cell line was grown as described (Palazuelos et al., 2011) in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% (v/v) foetal calf serum. Cells were grown in polyornithine-coated plates. HiB5 cell cultures were incubated in 5% CO_2 at 33 °C, the proliferation-permissive temperature of the oncogenic tsA58 allele of the SV40 large T antigen. Incubation at 39 °C results in loss of proliferative capacity and neural differentiation. In some experiments, HiB5 cells were pretreated for 30 min with SR141716 or SR144528 (2 μM) and then incubated with CBD (at the indicated doses) or WIN 55,212-2 (25 nM, Sigma) for 16 h. Stock solutions were prepared in dimethylsulfoxide. No significant influence of vehicle on any of the variables measured was observed at the final concentration used (0.1%, v/v). Control incubations included the corresponding vehicle content.

Proliferation and cell cycle analyses

HiB5 cells were pretreated with SR141716 and SR144528 (2 μM) for 30 min, cultured in the continuous presence of CBD (100 nM) for 16 h and, subsequently, with BrdU (100 μg/ml) for 30 min followed by immunostaining. For flow cytometry analysis HiB5 cells were trypsinized, permeabilized and fixed in 1% (w/v) of bovine serum albumin and 30% ethanol-PBS and labelled with 5 μg/ml Hoechst 33 342 (Invitrogen). Fluorescence intensity was analysed by using a LSR flow cytometer (Becton Dickinson, USA). Ten thousand cells per analysis were recorded. In some experiments, HiB5 cells were transiently transfected 1 d after plating with 1 μg pCIG2-FAAH-expressing vector or empty vector (Mulder et al., 2008) by using Lipofectamine 2000 following manufacturer's instructions (Invitrogen).

Data analysis

Data are presented as mean ± S.E.M. Significant differences between the groups were evaluated by *t* test, one, two or three-way analysis of variance (ANOVA) test, followed by Duncan's *post hoc* test. p values < 0.05 were considered significant.

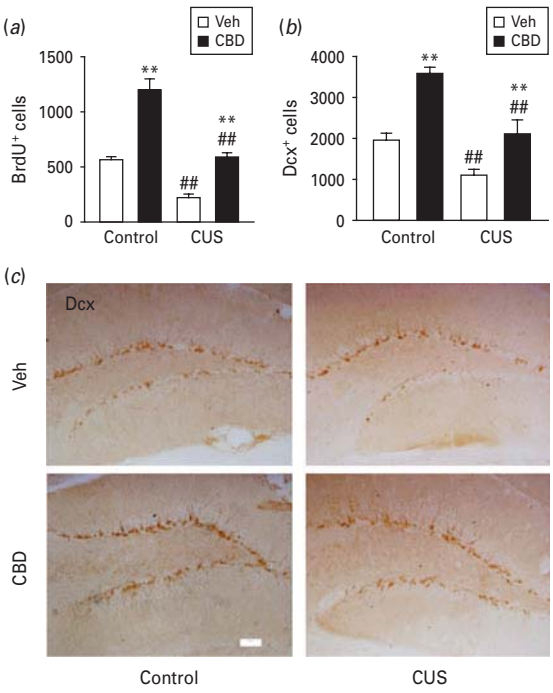

Fig. 1. Chronic unpredictable stress (CUS)-induced reduction of hippocampal neurogenesis is attenuated by cannabidiol (CBD) administration. (*a*) 5-bromo-2′-deoxyuridine (BrdU)-positive cells were quantified in the subgranular zone of the hippocampus in control non-stressed mice and in mice subjected to CUS that had received vehicle (Veh) or CBD (30 mg/kg; $n = 5$ per group). (*b*) Doublecortin (Dcx) immunoreactivity was quantified in the same groups of animals. (*c*) Representative images of Dcx immunohistochemistry are shown. Bar size, 100 μM. ** $p < 0.01$ *vs.* the respective vehicle-treated mice; ## $p < 0.01$ *vs.* the respective non-stressed mice ($p < 0.05$. Two-way analysis of variance followed by Duncan's *post hoc* test).

Results

CBD increases adult hippocampal neurogenesis and exerts an anxiolytic effect in a CUS model

To investigate the mechanism by which CBD exerts its anxiolytic effects and, in particular, its relation to hippocampal neurogenesis, we first exposed WT mice to a CUS model and CBD or vehicle was administered i.p. at 30 mg/kg for 14 d. CUS inhibited adult hippocampal neurogenesis as determined by quantification of BrdU-positive and doublecortin-expressing cells (Fig. 1*a–c*). CBD administration promoted hippocampal proliferation in control mice and counteracted the inhibitory effect of CUS in cell proliferation ($F_{1,19} = 154$, $p < 0.001$). Similarly, CUS induced a reduction of doublecortin$^+$ cells that was reversed by CBD administration ($F_{1,19} = 27$, $p < 0.001$). The increase in neurogenesis induce by CBD administration in WT mice was confirmed by a higher number of BrdU-positive newly born cells that expressed the mature neuronal marker NeuN (Fig 2*a*, *b*; *t* test $t_6 = 9.6$, $p < 0.001$). To determine if CBD exerts its anxiolytic effect via hippocampal neurogenesis, we employed

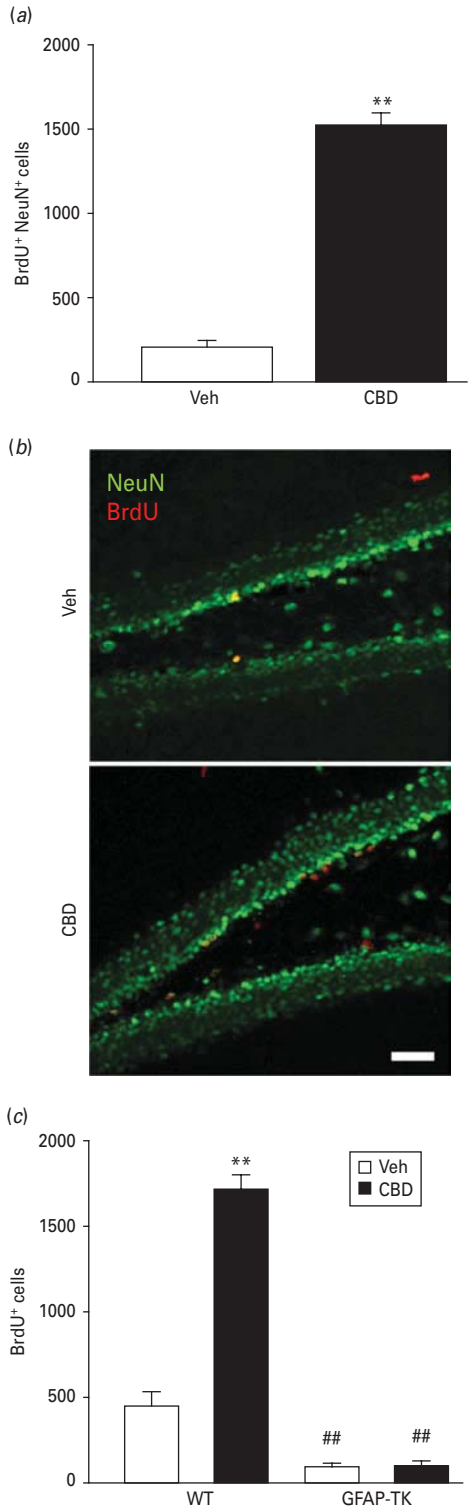

Fig. 2. Cannabidiol (CBD) administration exerts a proneurogenic effect. (*a*, *b*) Neurogenesis was determined by quantification of double-positive cells for 5-bromo-2′-deoxyuridine (BrdU) and NeuN in CBD- and vehicle (Veh)-treated wild-type (WT) mice. Representative immunofluorescence images are shown. Scale bar, 50 μM (*c*) BrdU-positive cells were quantified in the hippocampus of non-stressed WT and glial fibrillary acidic protein thymidine kinase (GFAP-TK) transgenic mice after Veh or CBD administration (30 mg/kg) for 14 d. Mice also received ganciclovir (100 mg/kg) for 7 d ($n=9$, 6, 8 and 8 animals per group, respectively). ** $p<0.01$ *vs.* the respective Veh-treated mice; ## $p<0.01$ *vs.* the respective WT mice ($p<0.05$ Two-way analysis of variance followed by Duncan's *post hoc* test).

Table 1. Home cage food consumption measured in transgenic GFAP-TK and WT littermates subjected or not to chronic unpredictable mild stress

Treatment	Genotype	Stress	Food (g)
CBD	WT	No	0.19 ± 0.00
Vehicle	WT	No	0.21 ± 0.01
CBD	GFAP-TK	No	0.20 ± 0.00
Vehicle	GFAP-TK	No	0.18 ± 0.00
CBD	WT	Yes	0.20 ± 0.00
Vehicle	WT	Yes	0.20 ± 0.00
CBD	GFAP-TK	Yes	0.17 ± 0.02
Vehicle	GFAP-TK	Yes	0.20 ± 0.05

GFAP-TK, Glial fibrillary acidic protein thymidine kinase; WT, wild type; CBD, cannabidiol.
Data are expressed as mean ± S.E.M.

GFAP-TK transgenic mice and their WT littermates. This approach allowed us to investigate the consequence of blocking adult hippocampal progenitor cell proliferation by GCV administration (Garcia et al., 2004; Palazuelos et al., 2009) on CBD anxiolytic action. Thus, GCV administration to GFAP-TK mice blunted CBD-induced hippocampal progenitor cell proliferation as determined by BrdU-positive cell quantification (Fig. 2*c*; $F_{1,12}=20.58$, $p<0.001$).

In non-stressed WT mice, CBD administration did not change NSF, but, in mice subjected to CUS, CBD exerted an anxiolytic-like effect by decreasing the latency to eat in the novel environment (Fig. 3*a*; $F_{1,47}=47.32$, $p<0.01$) without changing food intake in the home cage (Table 1). Analysis of the EPM test in stressed animals showed that in WT mice CBD promoted an anxiolytic-like effect by increasing the percentage of entries and time spent in the open arms (Fig. 3*b*; $F_{1,13}=8.13$, $p<0.05$). Similar to the NSF results, in the EPM, CBD effects were also prevented by hippocampal cell proliferation ablation in GFAP-TK mice. No effect in the number of enclosed-arm entries was found (Fig. 3*c*). These results evidenced that repeated CBD administration exerts an anxiolytic-like effect in mice subjected to CUS and that this occurs in parallel with changes in hippocampal neurogenesis. Importantly, blockade of adult neurogenesis prevented the anxiolytic effect of CBD on the NSF and EPM tests, therefore supporting the requirement of hippocampal neurogenesis in CBD actions.

CBD promotes hippocampal neurogenesis via CB_1 cannabinoid receptors by increasing anandamide levels

Considering the diversity of molecular targets that have been proposed to mediate CBD actions (Izzo et al., 2009),

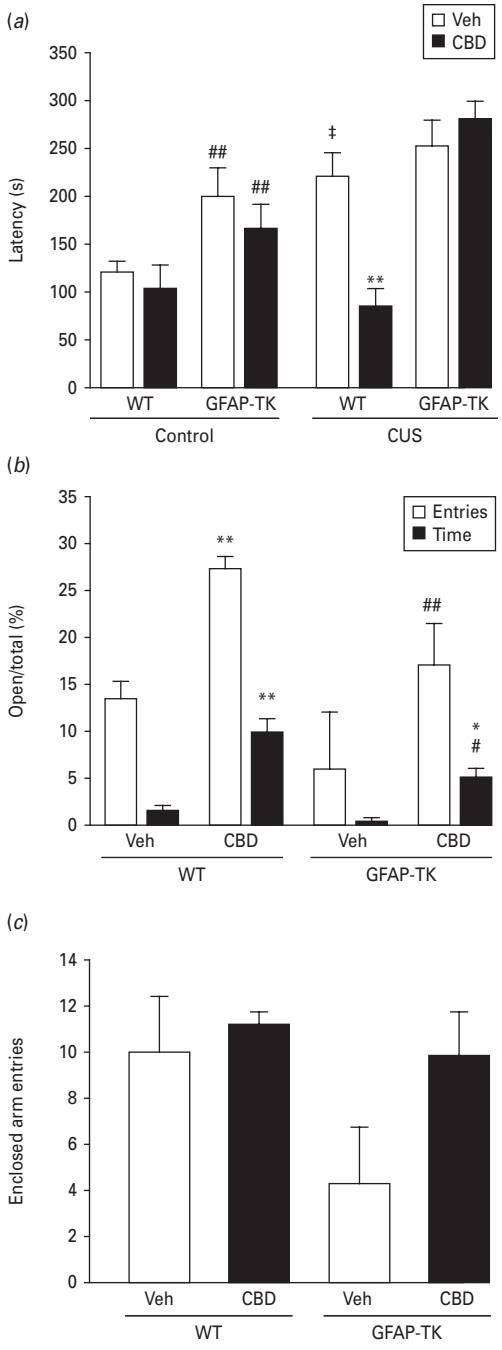

Fig. 3. Cannabidiol (CBD) administration exerts an anxiolytic effect in a chronic unpredictable stress (CUS) paradigm. (*a*) Wild-type (WT) and glial fibrillary acidic protein thymidine kinase (GFAP-TK) transgenic mice were subjected to CUS or left undisturbed and time latency in the novelty suppression feeding test was determined after chronic administration of vehicle (Veh) or 30 mg/kg CBD ($n = 14$, 12, 6, 7, 5, 4, 3 and 4 animals per group, respectively). (*b*, *c*) The anxiolytic effect of CBD administration in mice subjected to CUS was also determined in the elevated plus-maze test. White bars represent the percentage of entries into the open arms; black bars represent the percentage of the time spent in the open arms ($n = 4$, 3, 4 and 4 animals per group, respectively). Entries in the enclosed arms were also quantified (*c*). * $p < 0.05$, ** $p < 0.01$ *vs.* the respective Veh-treated mice; # $p < 0.05$, ## $p < 0.01$ *vs.* the respective WT mice; ‡ $p < 0.01$ *vs.* the respective non-stressed mice (two-way analysis of variance followed by Duncan's *post hoc* test).

and the involvement of CB_1 cannabinoid receptors regulating hippocampal neurogenesis (Aguado et al., 2005, 2007), we sought to investigate the potential involvement of the eCB system in CBD-induced neurogenesis and anxiolytic action. WT mice, subjected to CUS and administered with CBD or vehicle, were co-treated with either the CB_1 antagonist AM251 (1 mg/kg i.p. for 14 d) or vehicle. The anxiolytic-like effect of CBD in mice subjected to CUS in the NSF test was abrogated by AM251 administration (Fig. 4*a*; $F_{1,66} = 7.3$, $p < 0.01$). In the EPM test, there was a significant stress × drug treatment interaction ($F_{1,65} = 4.3$, $p < 0.05$). CUS decreased the percentage of open arm entries in control animals ($t_{15} = 2.39$, $p < 0.05$), an effect that was prevented by CBD. AM251 antagonized the increase in open-arm entries induced by CBD (Fig. 4*b*; $F_{1,65} = 4.3$, $p < 0.05$) in stressed animals, although on this occasion CBD administration did not increase the time spent in the open arms. No effect of CBD in the number of enclosed-arm entries was found (Fig. 4*c*) and there were no differences in non-stressed animals (Table 2). These results indicate that, although CBD is not believed to bind with high affinity to CB_1 receptors (Izzo et al., 2009), this receptor is involved in the CBD anxiolytic action observed here. We therefore evaluated if CBD modulates the eCB tone, as previously suggested, by inhibiting anandamide degradation (Bisogno et al., 2001; Leweke et al., 2012). Hippocampi from vehicle and CBD-treated mice were obtained and eCB levels were quantified by LC-MS. CBD-treated mice showed increased AEA levels, whereas 2AG and PEA were not affected (Fig. 4*d*).

CBD promotes neural progenitor proliferation via CB_1 and CB_2 cannabinoid receptors

To investigate the mechanism of action of CBD on neural progenitor cells, we used the HiB5 hippocampal progenitor cell line, which provides a good model to investigate the mechanism of action of cannabinoids as they express CB_1 and CB_2 cannabinoid receptors when cultured in proliferating conditions (Palazuelos et al., 2011). In addition, other potential mediators of CBD actions such as the vanilloid receptor TRPV1 and the serotonin $5\text{-}HT_{1A}$ receptor are also expressed in HiB5 cells (data not shown). Thus, HiB5 cells were exposed to increasing concentrations of CBD (50–500 nM) and quantification of BrdU-positive cells revealed that CBD promoted cell proliferation in a dose-dependent manner (Fig 5*a*; $F_{5,17} = 25.6$; $p < 0.001$). In addition, treatment of HiB5 cells with the CB_1/CB_2 receptor-mixed agonist WIN 55,212-2 (25 nM) and the eCB degradation inhibitors JZL184 (100 nM) and URB597 (60 nM) promoted progenitor cell proliferation (Fig. 5*b*). Hippocampal progenitors were next exposed to CBD (100 nM) together with CB_1 or CB_2 receptor-selective antagonists (SR141716 and SR144528, respectively, both at 2 μM) and CBD proliferative action was prevented (Fig. 5*b*,*c*). Based on previous reports (Campos and Guimaraes, 2008; Zanelati et al., 2010;

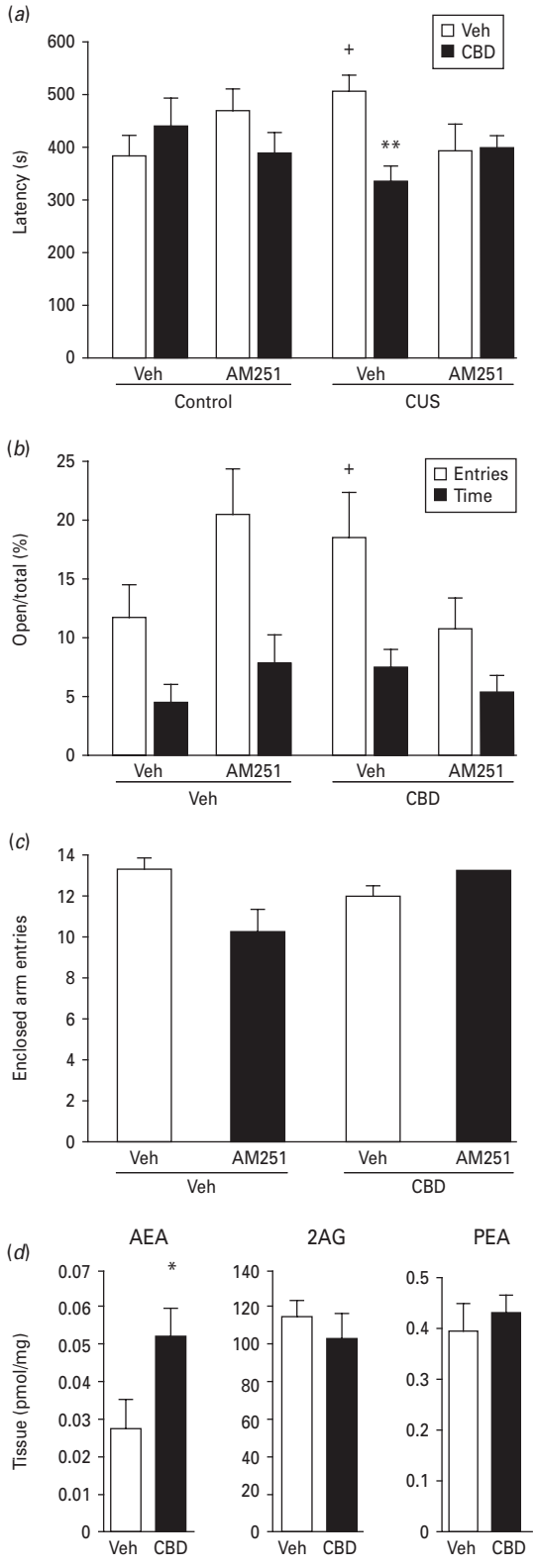

Fig. 4. The anxiolytic effect of cannabidiol (CBD) administration in chronic unpredictable stress (CUS) depends on CB_1 cannabinoid receptors. (*a*) Wild-type (WT) mice were subjected to CUS or left undisturbed and time latency in the novelty suppression feeding test was determined after chronic administration of vehicle (Veh) or 30 mg/kg CBD ($n=9$, 8, 10 and 10 animals per group, respectively). AM251 was administered (1 mg/kg) 10 min prior to CBD. (*b*, *c*) The anxiolytic effect of CBD administration in mice subjected to CUS was also determined in the elevated plus-maze test. White bars represent the percentage of entries into the open arms; black bars represent the percentage of the time spent in the open arms ($n=9$, 9, 10 and 10 animals per group, respectively). Entries in the enclosed arms were also quantified (*c*). (*d*) Endocannabinoid levels were determined in the hippocampus of mice treated chronically with Veh or CBD. PEA, Palmitoylethanolamide. * $p<0.05$, ** $p<0.01$ *vs.* the respective vehicle-treated mice; + $p<0.01$ *vs.* the respective non-stressed mice (analysis of variance followed by Duncan's *post hoc* test or Student's *t* test).

Table 2. Exploratory activity in the elevated plus maze of non-stressed mice treated for 14 d with vehicle or CBD (30 mg/kg.d) 10 min after AM251 (1 mg/kg.d) or vehicle

Treatment	Entries (%)	Time (%)	Enclosed entries
Vehicle	24.1±4.1	10.3±2.3	12.6±0.9
CBD	20.3±14.6	9.2±4.5	10.6±0.8
AM251	19.2±13.5	9.0±2.1	11.3±1.0
AM251+CBD	19.9±8.6	6.3±1.9	9.8±1.2

CBD, Cannabidiol.
Data are expressed as mean±S.E.M.

Gomes et al., 2011) we also determined the potential involvement of the 5-HT_{1A} receptor in CBD-induced progenitor cell proliferation. CBD treatment increased HiB5 cell number (Fig. 5*d*) and this effect was abrogated by the presence of the CB_1 and CB_2 antagonists, whereas the 5-HT_{1A}-selective antagonist WAY-100635 (2 μM) failed to prevent CBD-induced proliferation. The CB_2 antagonist alone exerted a paradoxical slight increase in cell proliferation. These results indicate that HiB5 cells express functional eCB receptors that can be activated indirectly by CBD and drive progenitor cell proliferation.

As CBD does not bind with high affinity to CB_1 or CB_2 receptors, but CBD-induced hippocampal progenitor proliferation and anxiolytic-like effects were blocked by CB_1 receptor antagonism, we tested if CBD could act on neural progenitors by interfering with the activity of the eCB-degrading enzyme fatty acid amide hydrolase (FAAH; Bisogno et al., 2001). To assess this possibility, we overexpressed FAAH in progenitor cultures to deplete their eCB tone (Mulder et al., 2008). HiB5 cell proliferation was thus determined in cells transfected with pCIG2-FAAH or empty vector and subsequently exposed to CBD. Quantification of BrdU-positive cells revealed that overexpression of the FAAH enzyme prevented the proliferative effect of CBD (Fig 5*e*; $F_{1,15}=20.3$, $p<0.001$). In addition, the proliferative effect of CBD was evaluated by flow cytometry analysis of DNA content after Hoeschst 33 342 staining. CBD treatment reduced the fraction of cells in the Go/G1 phase while increasing the fraction of cells in the S phase (Fig. 6*a*, *b*). This

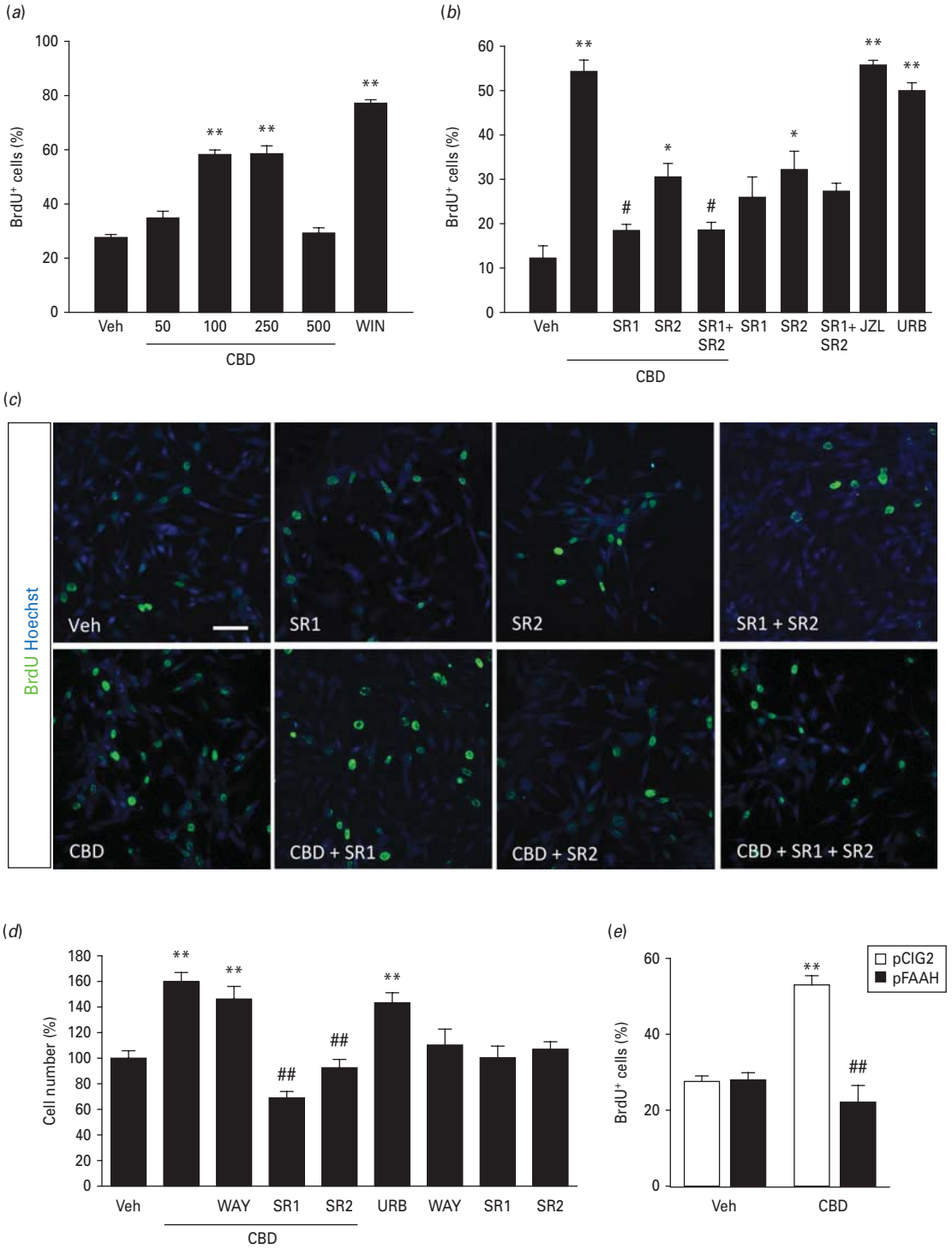

Fig. 5. Cannabidiol (CBD) promotes neural progenitor proliferation via CB_1 and CB_2 cannabinoid receptors. (*a*) Hippocampal HiB5 progenitors were treated with CBD at increasing concentrations (50, 100, 250 and 500 nM), WIN 55,212-2 (25 nM), URB597 (60 nM) or JZL184 (100 nM) for 18 h and 5-bromo-2′-deoxyuridine (BrdU)-positive cells were quantified after immunofluorescence and Hoechst 33 342 counterstaining. Results are provided as percentage of total cells. (*b*, *c*) The proliferative effect of CBD (100 nM) was determined as above in the presence of the CB_1 and CB_2 receptor antagonists SR141716 (SR1) and SR144528 (SR2), either alone or together. Representative images are shown. Bar size 60 μM. (*d*) Neural progenitors were treated with CBD (100 nM) for 48 h in the presence of SR1, SR2 or the 5-HT_{1A} antagonist WAY100235 (2 μM) and the number of cells was quantified in each condition. (e) HiB5 cells were transfected with pCIG2-fatty acid amide hydrolase (FAAH) or empty vector, treated with CBD (100 nM) or vehicle (Veh) and 5-bromo-2′-deoxyuridine (BrdU)-positive cells were quantified. Analysis of variance followed by Duncan's *post-hoc* test, * $p<0.05$, ** $p<0.01$ *vs.* the respective Veh-treated cells. # $p<0.05$, ## $p<0.01$ *vs.* the respective CBD-treated (*b*, *d*) or Veh-treated pCIG2-transfected cells (e). Results correspond to three independent experiments.

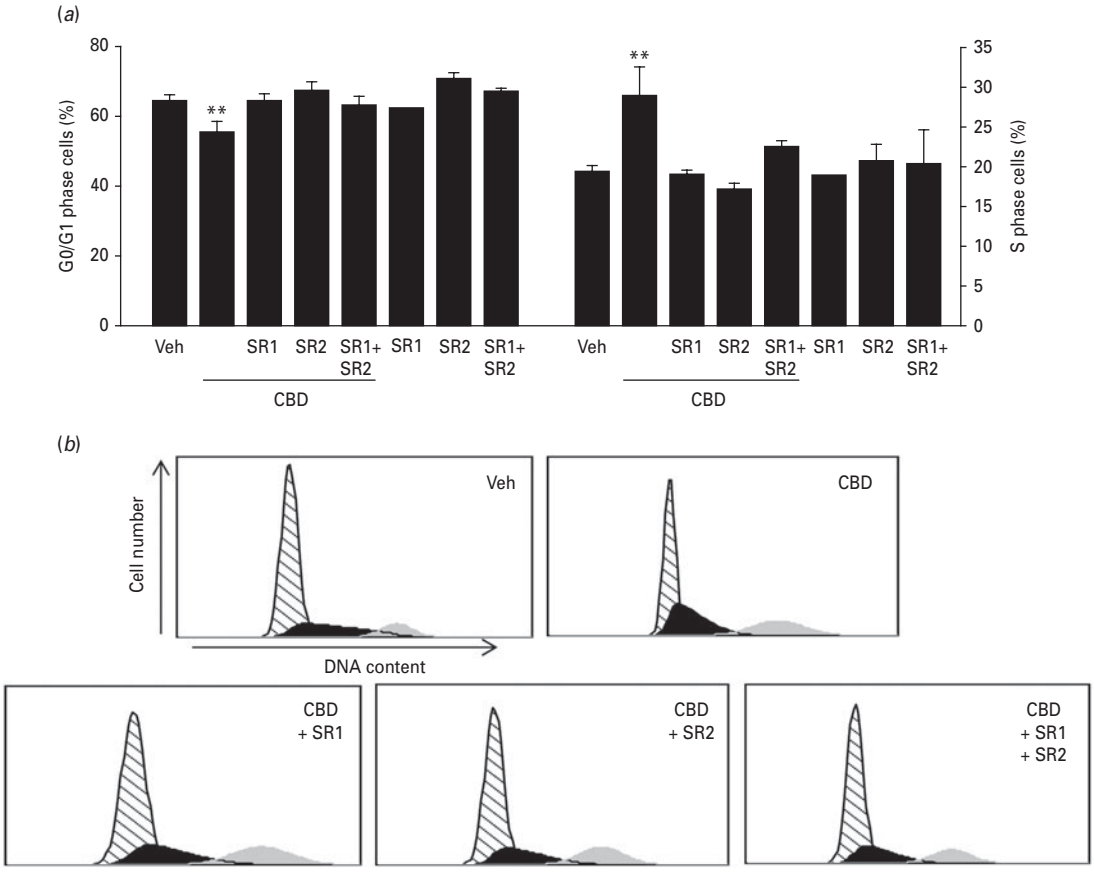

Fig. 6. Cannabidiol (CBD) promotes neural progenitor cell cycle progression at the G1/S transition in a CB_1 and CB_2 cannabinoid receptor-dependent manner. (*a*, *b*) HiB5 cells were treated with CBD (100 nM), alone or in the presence of SR141716 or SR144528 (SR1 or SR2, 2 μM) and cell cycle analysis was performed after DNA content quantification by flow cytometry. (*a*) The relative fraction of cells in the Go/G1 and S phases is shown. (*b*) A representative DNA histogram of each condition is shown. Results correspond to three independent experiments. One-way analysis of variance followed by Duncan's *post hoc* test, ** $p < 0.01$ *vs.* vehicle (Veh)-treated cells.

G1-S phase progression was prevented by SR141716 and SR144528. Overall, these results show that eCBs promote hippocampal progenitor proliferation and this effect can be mimicked by CBD, whose action relies on CB receptor engagement.

Discussion

The results shown herein contribute to the elucidation of the cellular and molecular mechanisms involved in the anxiolytic effect of chronic CBD administration. Specifically, genetic ablation of proliferating progenitors in the adult mouse brain prevents CBD anxiolytic action, thus demonstrating the requirement of hippocampal neurogenesis. In addition, CBD drives hippocampal progenitor cell proliferation *in vitro*, an effect that is abrogated by pharmacological blockade of CB_1 and CB_2 cannabinoid receptors or by overexpression of the eCB-degrading FAAH enzyme. Taken together, our findings strongly support that chronic CBD administration exerts an anxiolytic and proneurogenic hippocampal action by increasing the eCB tone.

Behavioural actions of CBD and neurogenesis

CBD is a plant-derived cannabinoid of high interest owing to its anxiolytic, antipsychotic and antidepressive actions evidenced in human studies as well as in animal models (Izzo et al., 2009). For example, CBD is effective for the management of some symptoms of schizophrenia and psychosis with less adverse effects than other antipsychotics (Leweke et al., 2012) and is also effective in social anxiety disorder. The beneficial effects of CBD administration in psychiatric symptoms adds to its safe profile in humans and the existence of CBD-containing standardized medicines (e.g. Sativex) and well-defined administration routes (e.g. oral and oro-mucosal). However, the mechanism of CBD action is complex and remains obscure, as many targets have been shown to be candidates for its behavioural actions. CBD can facilitate eCB-mediated neuromodulation by decreasing anandamide hydrolysis or re-uptake (Bisogno et al., 2001) and, among others, some of the anxiolytic effects of CBD are mediated by CB_1 receptors (Casarotto et al., 2010). Other acute anxiolytic and antidepressant effects of CBD seem to depend on facilitation of 5-HT_{1A}

receptor-mediated neurotransmission (Campos and Guimaraes, 2008; Gomes et al., 2011).

The present study supports that the proliferative effects of CBD on hippocampal progenitors are mediated by CB_1 and CB_2 receptors secondary to an increased eCB tone resulting from the inhibition of anandamide deactivation. However, CBD failed to modify PEA levels, which may be attributed to intrinsic differences in stability between AEA and PEA, the differential contribution of other acylethanolamide degrading enzymes (e.g. *N*-acylethanolamine-hydrolyzing acid amidase) and their different bulk levels. Our findings are in agreement with a recent study reporting that CBD-induced hippocampal neurogenesis is absent in CB_1-receptor knockout animals (Wolf et al., 2010) and the similarity between the effects of CBD and eCB-degradation inhibitors. Thus, like CBD, anandamide- and 2-arachidonoylglycerol-degradation inhibitors promote hippocampal progenitor proliferation and neurogenesis (Aguado et al., 2007) and exert beneficial anxiolytic effects while being devoid of undesired CB_1 receptor-associated psychoactivity (Kathuria et al., 2003; Busquets-Garcia et al., 2011; Kinsey et al., 2011). CBD, as well as other cannabinoids, produce typically bell-shaped dose–response curves, as seen here *in vitro* in proliferative experiments. Higher CBD concentrations can activate TRPV1 receptors and this effect has been associated by the lack of anxiolytic action observed with these doses (Campos and Guimaraes, 2009).

The importance of the eCB system, and, in particular, of CB_1 receptors, in mood control and depressive behaviours has been investigated for decades (Hill et al., 2009). Plant-derived cannabinoids exert a wide variety of effects on depressive and anxiety behaviours (Izzo et al., 2009). Alterations of the eCB system such as changes in CB_1 receptor expression and eCB levels are associated with major depression and suicide commitment (Hungund et al., 2004). An emerging paradigm from cannabinoid research is that the eCB system constitutes an allostatic signalling system that contributes to cellular plasticity responses in adaptation to stress-induced alterations (Patel and Hillard, 2008). Indeed, stress induces an inhibitory effect on neurogenesis that can be partially reverted by engaging the eCB system (Hill et al., 2006; present report). The role of adult neurogenesis in the regulation of cognition and mood is the object of intense study since the initial discovery of the adult hippocampal neurogenic niche (David et al., 2010; Deng et al., 2010). Blockade of hippocampal neurogenesis prevents some of the beneficial effects of antidepressant drugs and stimuli, although its ablation is not sufficient to induce depression and anxiety. However, blockade of adult neurogenesis makes mice more susceptible to stress-induced depressive behaviours (Snyder et al., 2011). Pharmacological manipulation and inducible genetic expansion of adult neurogenesis can improve depressive or anxiety-related behavioural changes (Santarelli et al., 2003; Sahay et al., 2011); likewise, proneurogenic stimuli such as environmental enrichment and running improve mood and cognition (Schloesser et al., 2010; Parihar et al., 2011). Thus, the emerging scenario indicates that adult hippocampal neurogenesis is involved in the plastic processes that allow for adaption to environmental changes (Dranovsky et al., 2011). Accordingly, by using transgenic GFAP-TK or hippocampus-irradiated mice, it has been demonstrated that inhibition of adult neurogenesis increases hypothalamic-pituitary-adrenal axis activity and glucocorticoid resistance (Snyder et al., 2011). The stress-induced anxiogenic response as determined in the NSF test is buffered by adult-born hippocampal neurons and, reciprocally, the neurogenic niche influences hippocampal progenitor cell fate (Dranovsky et al., 2011). Our results indicate that chronic CBD administration, by promoting neurogenesis, favours a similar anxiolytic response in stressed mice. The proneurogenic effect of CBD in non-stressed animals was not associated with behavioural changes in the NSF and EPM tests. This result agrees with previous reports showing that adult neurogenesis does not alter NSF behaviour under baseline conditions (Snyder et al., 2011), thus suggesting that hippocampal neurogenesis, rather than simply controlling emotional behaviours, favours adaptation and resilience to stress (Dranovsky et al., 2011; Snyder et al., 2011). Different from previous reports (Guimarães et al., 1990; Campos and Guimarães, 2008; Gomes et al., 2011), CBD did not induce any anxiolytic effect in non-stressed animals. In our study, however, the animals were tested 24 h after drug injection. This suggests that repeated CBD administration prevents the effects of CUS rather than induces an acute anxiolytic effect.

Role of cannabinoid receptors in proneurogenic stimuli

Antidepressive stimuli such as environmental enrichment and voluntary wheel running exert a proneurogenic action that has been shown to depend on the presence of functional CB_1 receptor signalling (Hill et al., 2010; Wolf et al., 2010). Voluntary running increased CB_1 receptor binding sites as well as anandamide levels in the hippocampus, but not in the prefrontal cortex, and administration of the CB_1 antagonist AM251 prevented running-induced proliferation (Hill et al., 2010). These results are in agreement with the ability of chronic CB_1 receptor activation to increase hippocampal progenitor proliferation and neurogenesis, which is associated with an anxiolytic/antidepressive cannabinoid action (Jiang et al., 2005). Aging-associated CA1 and CA3 neuronal loss and cognitive impairment are exacerbated in CB_1 receptor-deficient mice (Bilkei-Gorzo et al., 2005). Thus, similar to the beneficial anxiolytic action of chronic CBD in a CUS model, ageing-associated decline of neurogenesis can be partially prevented by the CB_1/CB_2 receptor-mixed agonist WIN 55,212-2, a beneficial action

that relies on the modulation of neuroinflammation (Marchalant et al., 2009a) and progenitor mobilization (Marchalant et al., 2009b). The role of CB_1 receptors in hippocampal neurogenesis, however, could be more complex, since spatially and locally restricted eCB signalling induction by CBD is proneurogenic, THC failed to promote or even inhibited adult neurogenesis (Wolf et al., 2010). This latter effect may be related to the spatial learning impairments caused by THC, an effect that is absent in animals treated with CBD (Fadda et al., 2004). In agreement with the hypothesis that the anxiolytic effect of repeated administration of CBD in the CUS model is mediated by the pro-neurogenic action of the CB_1 receptor, pharmacological blockade of this receptor blunted the behavioural effect of CBD.

CB_2 cannabinoid receptor agonists are also suitable candidates to promote neural progenitor proliferation (Palazuelos et al., 2006, 2011; Goncalves et al., 2008), although the consequences of this CB_2 receptor-evoked progenitor expansion in neurogenesis are as yet unknown. Ageing-associated decline of hippocampal and olfactory bulb neurogenesis can be prevented by the CB_2 receptor-selective agonist JWH-133 (Goncalves et al., 2008). Brain CB_2 receptors have recently been suggested to exert anxiolytic effects (Busquets-Garcia et al., 2011) and may be involved in social play behaviour by influencing post-natal neurogenesis in the amygdala (Krebs-Kraft et al., 2010). Thus, CBD can exert a dual action via CB_2 receptors acting directly in undifferentiated progenitor cells (Palazuelos et al., 2011) or indirectly through its immunosuppressive actions (Marchalant et al., 2009a). In addition, CBD administration protected from β-amyloid peptide-induced neuroinflammation and increased doublecortin-positive cells by a mechanism involving PPARγ receptors (Esposito et al., 2011). Anxiolytic- or antidepressant-like effects of single CBD administration in several animal models are prevented by the 5-HT_{1A} receptor antagonist WAY-100635 (Campos and Guimarães, 2008; Zanelati et al., 2010; Gomes et al., 2011). In the present study, however, 5-HT_{1A} antagonism failed to prevent CBD-induced proliferation, whereas CB_1 or CB_2 antagonists completely abrogated this response. Thus, although a partial contribution of 5-HT_{1A} receptors cannot be ruled out, CBD-induced proliferation seems to be largely mediated via cannabinoid receptors.

In conclusion, it is likely that, at least in part, some of the distinctive psychoactive effects of plant-derived cannabinoids in anxiety and depression (Izzo et al., 2009) may be due to their different regulatory properties on adult neurogenesis. The therapeutic potential of non-psychoactive cannabinoids in anxiety and depression, and in particular the anxiolytic effect of CBD, opens the door for their use to manage psychiatric symptoms in disorders such as ageing, stress and neuroinflammation, in which the neurogenic niche is affected.

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Statement of Interest

None.

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Review

Endocannabinoids via CB_1 receptors act as neurogenic niche cues during cortical development

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During brain development, neurogenesis is precisely regulated by the concerted action of intrinsic factors and extracellular signalling systems that provide the necessary niche information to proliferating and differentiating cells. A number of recent studies have revealed a previously unknown role for the endocannabinoid (ECB) system in the control of embryonic neuronal development and maturation. Thus, the CB_1 cannabinoid receptor in concert with locally produced ECBs regulates neural progenitor (NP) proliferation, pyramidal specification and axonal navigation. In addition, subcellularly restricted ECB production acts as an axonal growth cone signal to regulate interneuron morphogenesis. These findings provide the rationale for understanding better the consequences of prenatal cannabinoid exposure, and emphasize a novel role of ECBs as neurogenic instructive cues involved in cortical development. In this review the implications of altered CB_1-receptor-mediated signalling in developmental disorders and particularly in epileptogenesis are briefly discussed.

Keywords: cortical progenitor; neurogenesis; endocannabinoid signalling

1. INTRODUCTION

The developing nervous system is characterized by highly active and dynamically regulated cellular processes involving cell generation and differentiation, migration to their final destination, neuronal maturation and establishment of appropriate neuronal connectivity [1,2]. The precise regulation of these processes is achieved by a complex network of intrinsic molecular determinants and intracellular signalling pathways that are in turn modulated by surrounding information from the neurogenic niche [3]. Numerous studies have begun to delineate some of these determinants and signalling pathways involved in neural cell fate decisions, such as the regulatory switch responsible for neuronal versus glial differentiation [4] and the specification of dorsal (pallial) versus ventral (subpallial) neurons [3]. Developmental neurobiology studies and advances in stem cell research have allowed the identification of some of the molecular mechanisms involved in the specification and differentiation of specific neuronal lineages with different neurotransmitter phenotypes (e.g. glutamatergic, GABAergic, dopaminergic, etc.) [5]. However, the precise extracellular signalling pathways that modulate the acquisition of the diversity of developing neuronal populations and guarantee their appropriate integration are still only partially understood. Exposure of the developing and maturing nervous system to marijuana-derived cannabinoids exerts a significant impact on behavioural aspects, particularly regarding the control of emotions and cognitive responses. The implications of cannabinoid exposure in human neuropsychiatric disorders (see other reviews in this special issue and [6,7]) have driven the investigation on the mechanism of action and neurobiological substrate underlying developmental action of cannabinoids. Endocannabinoids (ECBs) have recently been underscored as neurodevelopmental signalling cues that, by targeting the CB_1 cannabinoid receptor, exert a regulatory role on the molecular and cellular mechanisms involved in brain development. Here, we review the experimental evidence supporting the functional role of the ECB system during cortical development, as derived from genetic and pharmacological manipulation studies. The CB_1 receptor has emerged as a novel signalling platform that drives neuronal generation and specification, thereby modulating brain maturation and connectivity. We also discuss the potential implications of these findings in proper neuronal activity of the adult brain.

2. THE ENDOCANNABINOID SYSTEM IN THE DEVELOPING BRAIN

The expression pattern of the ECB system elements (including receptors and enzymes of synthesis and degradation) in the developing brain has been addressed, revealing the presence of diverse ECB-metabolizing

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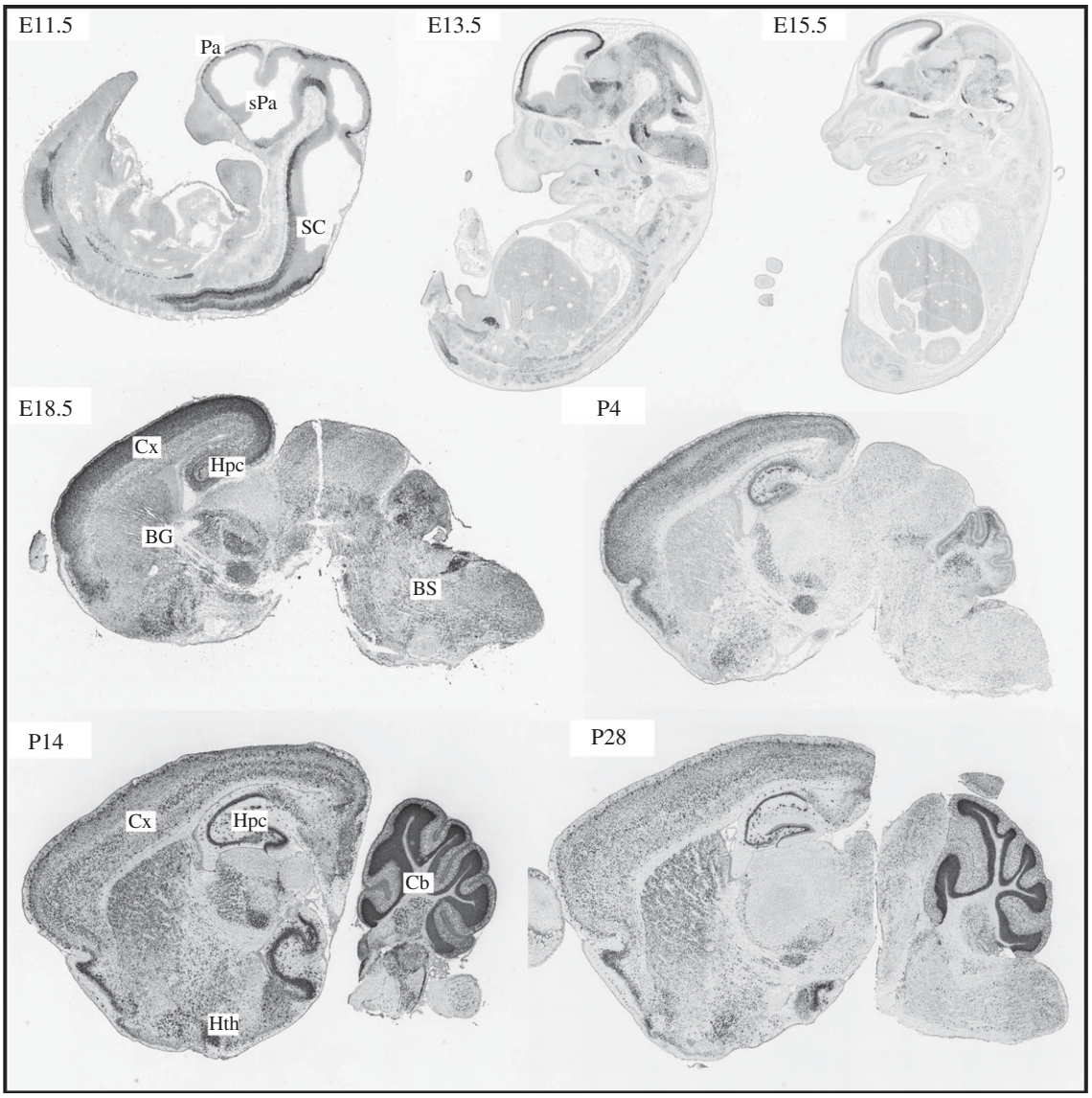

Figure 1. Expression pattern of the CB_1 cannabinoid receptor mRNA at different developmental stages. CB_1 mRNA *in situ* hybridization in the developing mouse nervous system is shown at the indicated stages. BG, basal ganglia; BS, brainstem; Cx, cortex; Hpc, hippocampus; Hth, hypothalamus; Pa, pallium; sPa, subpallium; SC, spinal cord. Published with permission of Allen Developing Mouse Brain Atlas, Seattle (WA), Allen Institute for Brain Science. Copyright ©2009. Available at: http://developingmouse.brain-map.org.

enzymes with restricted subcellular and spatio-temporal distribution. This complexity and the rapid rates of ECB synthesis/degradation reveal the existence of a dynamically regulated ECB tone during active neurogenesis. We will focus here on the expression pattern of the CB_1 receptor as the most important molecular target of the ECB tone [8]. The CB_1 receptor is expressed from very early stages of embryonic development, even before the appearance of the neural tube and neuroectoderm development. CB_1 is present in trophoblast stem cells and its deletion results in reduced cell proliferation and differentiation that is followed by aberrant placentation and compromised embryo implantation [9]. In addition to CB_1 receptor expression in the blastocyst stage, the other G-protein-coupled cannabinoid receptor, the CB_2 receptor, is also present in the inner cell mass, and has been proposed to be involved in embryonic stem-derived haematopoietic cell proliferation and lineage differentiation [10]. Cannabinoid administration during chick gastrulation results in alterations of neural tube formation and patterning, thus revealing the early sensitivity of the developing nervous system to cannabinoid signalling interference [11].

In mammals, CB_1 receptor expression during neural development is characterized by its abundant levels in white matter areas, with their levels progressively increasing from prenatal stages to adulthood in grey matter areas [12]. This atypical distribution of CB_1 receptor expression during development occurs while active neurogenesis and axonal migration occurs and prior to synaptic maturation and neuronal activity. Therefore, neurodevelopmental CB_1 receptor actions are likely to be independent of their regulatory role of neurotransmitter release and neuronal activity. The CB_1 mRNA expression pattern in the developing mouse brain is summarized in figure 1. CB_1 is present in the telecenphalon from E11.5 and its early expression is also observed in the developing spinal cord. During cortical development, CB_1 receptors are present in pioneer neurons that populate the marginal zone of the

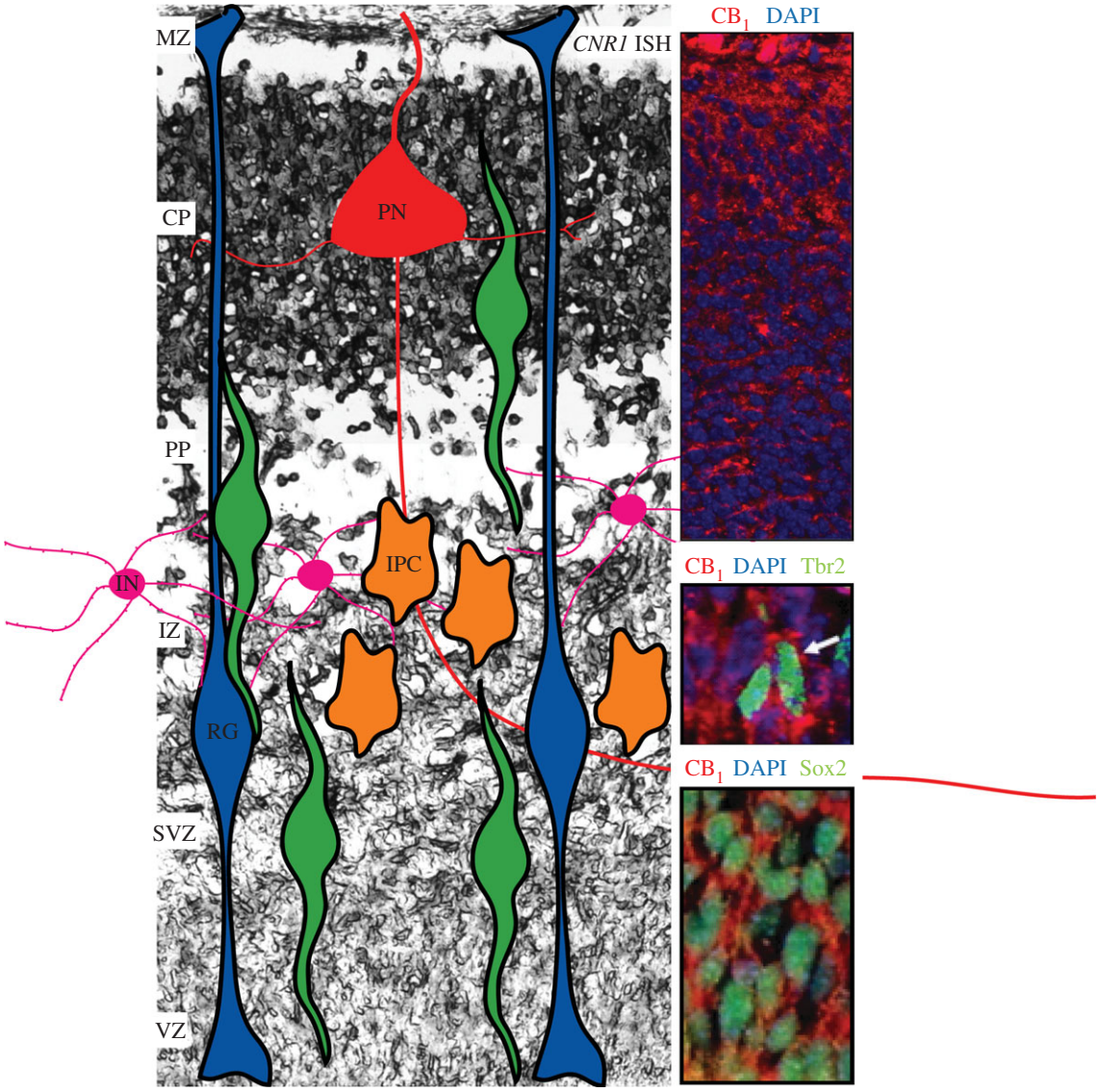

Figure 2. CB_1 cannabinoid receptor expression during cortical development. The CB_1 receptor is present in the developing cortex, showing increasing expression levels from undifferentiated to differentiated projection neurons (PNs). The CB_1 receptor is present in Cajal–Retzius cells of the marginal zone (MZ) and apical and basal progenitors in the ventricular and subventricular (VZ/SVZ) proliferative area. Representative immunofluorescence images showing the colocalization of the CB_1 receptor in radial glial (RG) progenitors and intermediate amplifying progenitor cells (IPCs) as identified with Sox2 and Tbr2 antibodies, respectively [13] (copyright National Academy of Sciences, USA 2009). Higher expression levels of the CB_1 receptor are evident in maturing neurons that have reached the CP, that correspond to locally generated PNs. CB_1 receptor is present in certain interneuron (IN) populations that reach the pallium upon tangential migration from the ganglionic eminences. Image background corresponds to a representative *in situ* hybridization of the *CNR1* mRNA at E.16.5 (by C. Hoffman and B. Lutz, Johannes Gutenberg University Mainz, Germany).

dorsal cortex (figure 2). In particular, CB_1 is present in Cajal–Retzius cells (E12.5) that are characterized by reelin expression [14,15]. Reelin is well known for its role as an instructive signalling cue that, among other actions, promotes radial migration of differentiating neurons. At embryonic day E13.5–E14.5, mouse developing cortex shows higher CB_1 receptor expression in the intermediate zone and developing cortical plate (figure 2), where postmitotic neuroblasts and differentiating neurons are located, and expresses early neuronal markers such as class III β-tubulin [13,14]. At these stages, the CB_1 receptor is also present in the subpial area of the ganglionic eminences and the primordium of the hippocampus [15]. Later, CB_1 receptors are heterogeneously distributed through cortical layers and the hippocampus, in both excitatory glutamatergic projection neurons, as identified by vGlut1 expression, and cholecystokinin (CCK)-expressing GABAergic interneurons colabelled with vGlut3 [16–18]. $CB_1^+CCK^+$ interneurons derived from the ganglionic eminences follow tangential migratory routes from the ventral telencephalon and reach the developing cortex, hippocampus and amygdala [15,19–21]. The regulatory role of the ECB system in development of excitatory and inhibitory neuronal lineages is also conserved in the adult brain, in which CB_1 receptors are functional in cortical excitatory projecting neurons and inhibitory GABAergic interneurons [8,22].

The expression and functionality of the ECB system has also been characterized in human brain development [23,24]. In human foetal brain, *in situ* hybridization and binding assays evidence a heterogeneous pattern of CB_1

receptor expression with preferential limbic expression and high levels throughout the cerebral cortex, hippocampus, caudate nucleus, putamen and cerebellum. CB_1 receptors are present at gestational week 9 in the subventricular zone (SVZ) and Cajal–Retzius cells of the marginal zone [25]. In the second trimester of gestation, intense labelling for CB_1 receptors is evident in the hippocampal CA region [24]. High densities of CB_1 receptors are detected during prenatal development in fibre-enriched areas that later in the adult brain are practically devoid of these receptors [23]. Overall, the early expression and functionality of the CB_1 receptor during nervous system development and its transient and atypical localization in prenatal stages suggest a specific role of the ECB system in human brain development, with potential implications in neuropsychiatric disorders [6,26].

3. THE CB_1 CANNABINOID RECEPTOR IN NEURAL STEM/PROGENITOR CELLS

Neural stem and progenitor cells of different embryonic brain areas express a functional ECB system. ECBs are actively produced in the neurogenic niche of the developing cortex and engage CB_1 receptors on NPs of the ventricular zone (VZ; figure 2), as identified by the expression of the neuroepithelial marker nestin and the transcription factor Sox2 [13,27]. Intermediate progenitor cells of the SVZ, characterized by the expression of the transcription factor Eomes/Tbr2, that contribute to the generation of pyramidal cells in all layers of the cerebral cortex [28], are also targeted by CB_1 receptors (Díaz-Alonso *et al.* 2012, unpublished results). CB_1 receptors are present in dividing cells identified by 5-bromo-2′-deoxyuridine labelling, the expression of endogenous cell cycle markers (Ki-67, phosphorylated-histone 3) and the phosphorylation of vimentin (a marker of radial progenitor cell division) [13,29,30]. These observations indicate that the CB_1 receptor present in both apical radial progenitors and basal intermediate progenitor cells, albeit at low expression levels when compared to differentiated neurons, exerts a regulatory role in progenitor cell fate. Whereas in the developing chick embryonic CB_1 receptor expression follows neuronal differentiation and, at least in the spinal cord, might be restricted to postmitotic neurons [31,32], its expression pattern in the nervous system of the zebrafish is suggestive of its involvement in neurogenesis [33].

Neurospheres (non-adherent *in vitro* culture of NP cells) from embryonic and postnatal development stages express CB_1 receptors and the anandamide (AEA)-degrading enzyme fatty acid amide hydrolase (FAAH), and elevations in their intracellular Ca^{2+} concentration increase ECB production [29]. In addition, the CB_{2-} receptor and diacylglycerol lipase (DAGL), the enzyme responsible for 2AG generation, are also functional in NP cultures [34,35]. AEA and 2-arachidonoylglycerol (2AG) can act therefore in an autocrine or paracrine manner on NPs or surrounding neighbour cells. DAGL expressed at embryonic stages is preferentially located in axon growth cones and is later redistributed to dendrites where it controls the 2AG retrograde neuromodulatory signalling role [36]. During corticogenesis, as well as in the developing retina, ECB production by *N*-acyl phosphatidylethanolamine–phospholipase D (NAPE-PLD; one of the enzymes responsible for AEA generation) and DAGL participate in axon guidance [37,38]. *In vitro* studies in neuroblastoma cells confirmed the positive action of 2AG production in neurite outgrowth and the existence of different mechanisms of action according to the metabolic origin of 2AG [39]. Unfortunately, the precise contribution of the two DAGL enzyme isoforms (α and β) in neural development remains to be clarified (see accompanying paper by Doherty *et al.*). In the adult, SVZ DAGLα is present in ependymal cells that are intimately related to neural stem cells, and mediates 2AG generation involved in the regulation of neurogenesis [40]. The analysis and characterization of the DAGL locus identified the minimal core promoter sequence and the involvement of the transcriptional regulator specificity protein Sp1 in DAGLα expression. High expression levels of DAGLα in the NSC line Cor-1 rapidly decrease through their differentiation into GABAergic neuronal cells [41], whereas in neuroblastoma cells retinoic acid-induced neuronal-like differentiation increases first DAGLα expression and later DAGLβ [39]. In the developing forebrain, monoacylglycerol lipase (MGL) expression is preferentially observed in the thalamus, thus restricting local 2AG levels and relieving non-permissive axonal growth of corticothalamic projections [42].

Although not discussed here in detail, the regulatory role of the CB_1 receptor in neuronal generation and maturation in the embryonic brain is preserved in the neurogenic niches of the adult brain. NPs in adult neurogenic brain areas also express the CB_1 receptor and produce ECB ligands [29,30,43]. CB_1 receptors are expressed in NP cells of the subgranular zone (SGZ) and SVZ, in which they drive progenitor proliferation and tune neural differentiation. These findings indicate that the role of ECBs as developmental signalling cues is conserved in the mature nervous system [44].

(a) *The CB_1 cannabinoid receptor drives neural progenitor cell proliferation*

CB_1 receptor activity in NPs regulates cell proliferation and survival. *In vitro*, the use of neurosphere cultures of embryonic cortical NPs derived from knockout mice has shown that inactivation of the CB_1 receptor, as well as of the CB_2 receptor, reduces cell proliferation and impairs self-renewal [29,34]. Accordingly, pharmacological regulation with selective CB_1 and CB_2 receptor agonists or antagonists exerts a positive or negative action, respectively, on NP cell division [29,30,34,40,45]. *In vivo*, CB_1 receptor loss of function induces alterations of cortical and hippocampal development [20,29] and, whereas CB_1-null mice have reduced cortical progenitor proliferation, in FAAH-deficient mice the opposite is observed [13,29]. Abnormal cortical development in CB_1-deficient mice is characterized by defective SVZ/VZ pyramidal progenitor proliferation and radial migration, deficits in axonal navigation and aberrant corticofugal projections [13]. The role of the ECB system in the regulation of pyramidal NP cell expansion during cortical development is also recapitulated in brain slices, in which pharmacological regulation of CB_1 receptors or genetic

manipulation of the ECB tone disrupts proper pyramidal neuron generation [13]. NP proliferation from other brain areas such as the cerebellum is also dependent on CB_1 receptor activation [45].

4. CANNABINOID SIGNALLING IN NEURAL PROGENITOR/STEM CELLS

CB_1 receptor signalling in neural cells has been extensively studied, but the existence of selective CB_1 receptor-mediated signalling mechanisms in progenitor cells remains to be investigated in detail. CB_1 receptor-evoked signal transduction pathways can be divided into two large categories: canonical signalling via the classical repertoire of heterotrimeric Gi protein partners, and crosstalk with other membrane receptor-dependent signalling events (in particular those elicited by neurotrophin/growth factor receptors). Current understanding of the signal transduction mechanisms regulated by CB_1 receptors in NPs is summarized in figure 3.

(a) *CB_1 cannabinoid receptor signalling mechanism and cell proliferation*

The CB_1 receptor-mediated proliferative and pro-survival actions have been attributed, at least in part, to the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt axis and extracellular signal-regulated protein kinase (ERK; figure 3) [48]. The CB_1 receptor, via canonical Gi-mediated inhibition of adenylyl cyclase, decreases cAMP concentration, and this in turn plays a prominent role by de-inhibiting the ERK pathway by protein kinase A [49,50]. In addition, G protein βγ subunits liberated upon CB_1 receptor activation stimulate the ERK pathway in a PI3K-dependent manner [51]. Therefore, both regulation of cAMP levels and PI3K signalling contribute to CB_1-mediated ERK activation. However, the mechanisms of CB_1 receptor-mediated ERK activation are multiple and interconnected, thus providing a rather complex scenario. It is likely that, at different time points, upon CB_1 receptor activation ERK activation may occur by different mechanisms [52]. According to this model, early ERK activation would be strongly dependent on cAMP levels, activation of members of the cytosolic tyrosine kinase Src family and transactivation of tyrosine kinase receptors. In cerebellar granular progenitor cells, CB_1 receptor coupling to the PI3K/Akt pathway is followed by the activation of the glycogen synthase kinase-3β/β-catenin pathway [45]. CB_1 receptor activation therefore increases β-catenin nuclear localization and the activation of lymphoid enhancer factor/T-cell factor transcription factors induces proliferation, thereby modulating cell cycle regulatory genes such as cyclin D1.

CB_1 signalling in neural cells may also involve the activation of mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine protein kinase that regulates cell growth, proliferation and survival [53]. CB_1 receptor stimulation in hippocampal GABAergic neurons activates mTORC1 and downstream p70S6K in pyramidal neurons that, by controlling protein synthesis, is responsible for some amnesic effects of Δ^9-tetrahydrocannabinol administration [54,55]. Therefore, CB_1 receptor-induced mTORC1 and protein synthesis regulation can explain some long-term cannabinoid actions on neuronal plasticity and cognition. The role of CB_1 receptors in mTORC1 signalling during brain development remains unknown, although CB_2 receptors have recently been shown to be coupled to mTORC1 activation in NP cells both in the developing cortex and in the SGZ of the adult hippocampus [56]. At postnatal stages, mTORC1 signalling is known to be involved in oligodendrocyte differentiation and myelination [53,57], and the ECB system drives oligodendroglial differentiation and cell survival at least partially via mTORC1 regulation [58]. In contrast to neurons and progenitor cells, in which mTORC1 is activated by cannabinoid receptors [54], in transformed glioma cells cannabinoids, via tribbles homologue 3, inhibit the Akt/mTORC1 axis and can

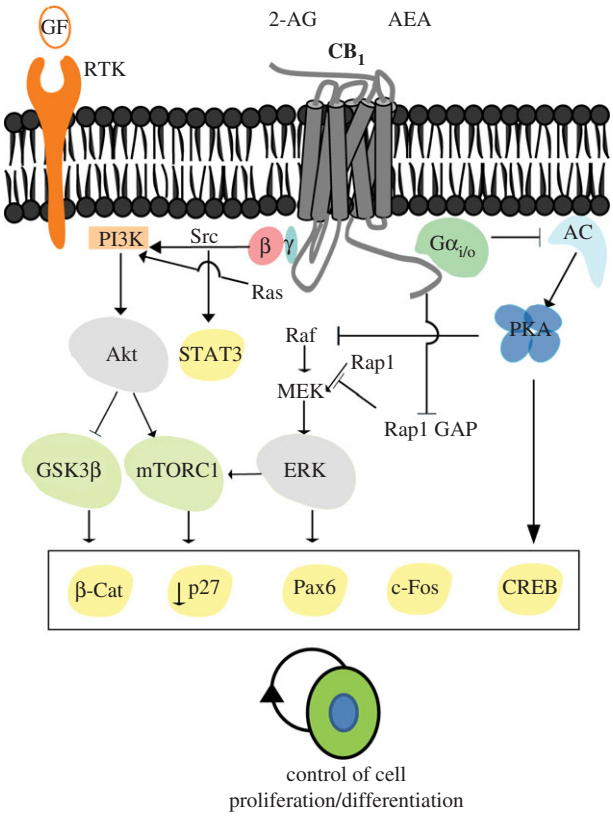

Figure 3. CB_1 cannabinoid receptor signalling and regulation of neural stem/progenitor cell proliferation. CB_1 receptors are coupled to Gi proteins, thereby mediating the inhibition of adenylyl cyclase (AC) and protein kinase A (PKA). CB_1 receptor coupling to Gi signalling is also associated with activation of the extracellular signal-regulated kinase (ERK) pathway via different mechanisms (see text for details). Direct activation of the PI3K/Akt and ERK pathways by CB_1 receptors may converge, thus synergizing with their activation by other receptors such as growth factor receptors with tyrosine kinase activity (RTK). CB_1 receptor-induced activation of RTKs can occur by promoting the processing of membrane-bound growth factor inactive precursors to yield active growth factors, or by activating intracellular Src family protein kinases. In some circumstances, CB_1 activity can antagonize RTK-mediated ERK signalling (see [46,47] for further details). Activation of the CB_1 receptor ultimately controls different transcriptional regulators, including CREB, STAT-3, PAX-6 and β-catenin. The CB_1 receptor may also regulate mammalian target of rapamycin complex 1 (mTORC1) in NPs as it occurs in differentiated neurons.

switch on an autophagy programme that results in cell death by apoptosis [59,60].

How this diversity of intracellular CB_1 receptor signalling mechanisms in neural cells is regulated remains poorly understood. CB_1 receptors may form homo- or heterodimers with other G-protein-coupled receptors [61,62] and this may shift intracellular signalling coupling. Importantly, although CB_2 receptors share some of the CB_1 receptor signalling effectors (inhibition of cAMP production, ERK and PI3K/Akt activation), their opposite pattern of expression during NP cell differentiation (i.e. NPs are $CB_1^{low}CB_2^{+}$, while differentiated neurons are $CB_1^{+}CB_2^{neg}$) may lead to different ratios of homo- and heterodimers that can promote alternative cell fate decisions according to the major signalling pathway engaged.

(b) *CB_1 cannabinoid receptor crosstalk with other extracellular signalling pathways*

CB_1 receptors have been shown to crosstalk with growth factor and neurotrophin signalling events at different levels (figure 3). CB_1 receptor activation is associated to changes in growth factor expression, and can regulate tyrosine kinase growth factor receptors by direct transactivation mechanisms. In the adult nervous system, CB_1 receptor expression is involved in the regulation of the levels of the neurotrophin brain-derived neurotrophic factor (BDNF), and thus CB_1-deficient mice have reduced hippocampal BDNF levels under basal circumstances, which could explain some of the neuronal plasticity and emotional alterations shown in those animals [63–65]. Transactivation of growth factor receptors with tyrosine kinase activity (EGFR, Trk B and others) has been shown to be involved in some CB_1 receptor-mediated neurodevelopmental actions [52]. CB_1 receptor-induced transactivation can be mediated by growth factor or cytokine (e.g. TNFα) expression or their processing and shedding from inactive membrane-bound precursors [66,67]. Moreover, transactivation can occur via cytosolic tyrosine kinases of the Src family and this mechanism may influence interneuron migration [20]. Growth factor levels are also regulated by cannabinoid signalling under different neurodegenerative paradigms, such as hippocampal and striatal excitotoxicity, in which BDNF, fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) are tuned by CB_1 receptors [68–70]. Reciprocally, FGF receptors promote axonal growth and guidance via DAGL activation and 2AG generation [71].

CB_1 receptor activation can also lead to the regulation of small G proteins and subsequent control of cytoskeleton and microtubule dynamics, which may be responsible for cannabinoid actions on neuritogenesis and synaptogenesis. Activation of CB_1 receptors can induce either neurite outgrowth or retraction [72–76]. CB_1 receptors are enriched in the axonal growth cones of GABAergic interneurons at late gestation and, when activated, they induce a chemorepulsive collapse of axonal growth cones by activating RhoA [37,73]. CB_1 receptor-induced neurite outgrowth in neuroblastoma Neuro2A cells occurs via Rap1, Src and the signal transducer and activator of transcription 3 (STAT 3) [74,76]. CB_1 receptor activation and IL6 receptor signalling exert a synergistic effect in cAMP–responsive element binding protein (CREB) and STAT3 activation that enforces neurite outgrowth [77]. In the retina, the CB_1 receptor induces growth cone collapse in a mechanism involving the intracellular trafficking of the deleted in colorectal cancer receptor [38]. Nerve growth factor-induced neurite outgrowth of PC12 cells is inhibited by CB_1 receptor modulation of Trk A/Rap1/B-Raf-mediated sustained ERK activation [72]. The recent demonstration that recruitment of the Gi-interacting protein GRIN (G-protein-regulated inducer of neurite outgrowth) upon CB_1 receptor activation can determine the signalling output of FGF stimulation, by allowing Sprouty-mediated inhibition of ERK signalling [46], may reconcile the apparent conflicting results of CB_1 receptors mediating a positive or inhibitory action in neurite outgrowth and ERK activation. In summary, further investigation on the role of recently described CB_1 receptor interacting proteins (i.e. CRIP1, AP3 and others) will shed light on cannabinoid signalling mechanisms [78] and may clarify the different neurodevelopmental actions of CB_1 receptor activity. Importantly, the different kinetics and intensity of signal transduction pathways engaged by the CB_1 receptor in a particular cellular context can induce different NP cell fate decisions, for example from proliferation and self-renewal (acute ERK activation) to neural differentiation (sustained ERK activation).

5. THE CB_1 CANNABINOID RECEPTOR AND NEURAL DIFFERENTIATION

The diversity of neurodevelopmental actions of the CB_1 receptor is suggestive of a regulatory role of the ECB system in neural cell differentiation and morphogenesis. CB_1 receptor activity has been associated to the regulation of different neural cell types' development, including neurons and glial cells. Genetic elimination of the CB_1 receptor at embryonic stages induces alterations of long-range subcortical axonal projections, but the particular mechanisms responsible for this deficit in CB_1 knockout cells are as yet unknown and may include: (i) defective VZ/SVZ pyramidal progenitor cell proliferation; (ii) impairment of radial migration; (iii) neuronal differentiation alterations; and (iv) axonal pathfinding disturbance. Inhibition of 2AG synthesis reduced vGlut1 expression and altered the expression of the glutamatergic synapse markers SNAP25 and synaptophysin [13]. However, this finding alone does not prove a regulatory role of CB_1 receptors in neuronal differentiation. CB_1 receptor expression increases with neuronal cell differentiation and thus increased or reduced CB_1 expression are likely to occur in parallel with changes in the expression of other neuronal markers. Although at embryonic stages CB_1 receptor ablation results in reduced neurogenesis [29,30], at postnatal stages manipulation of the ECB system interferes with astrocyte and oligodendrocyte development [27,79,80]. In these studies, altered neural cell populations upon CB_1 signalling manipulation are observed concomitantly with reduced progenitor cell proliferation. These observations raise the question of whether the CB_1 receptor tunes lineage selection of undifferentiated cells or acts by merely expanding specific NP populations.

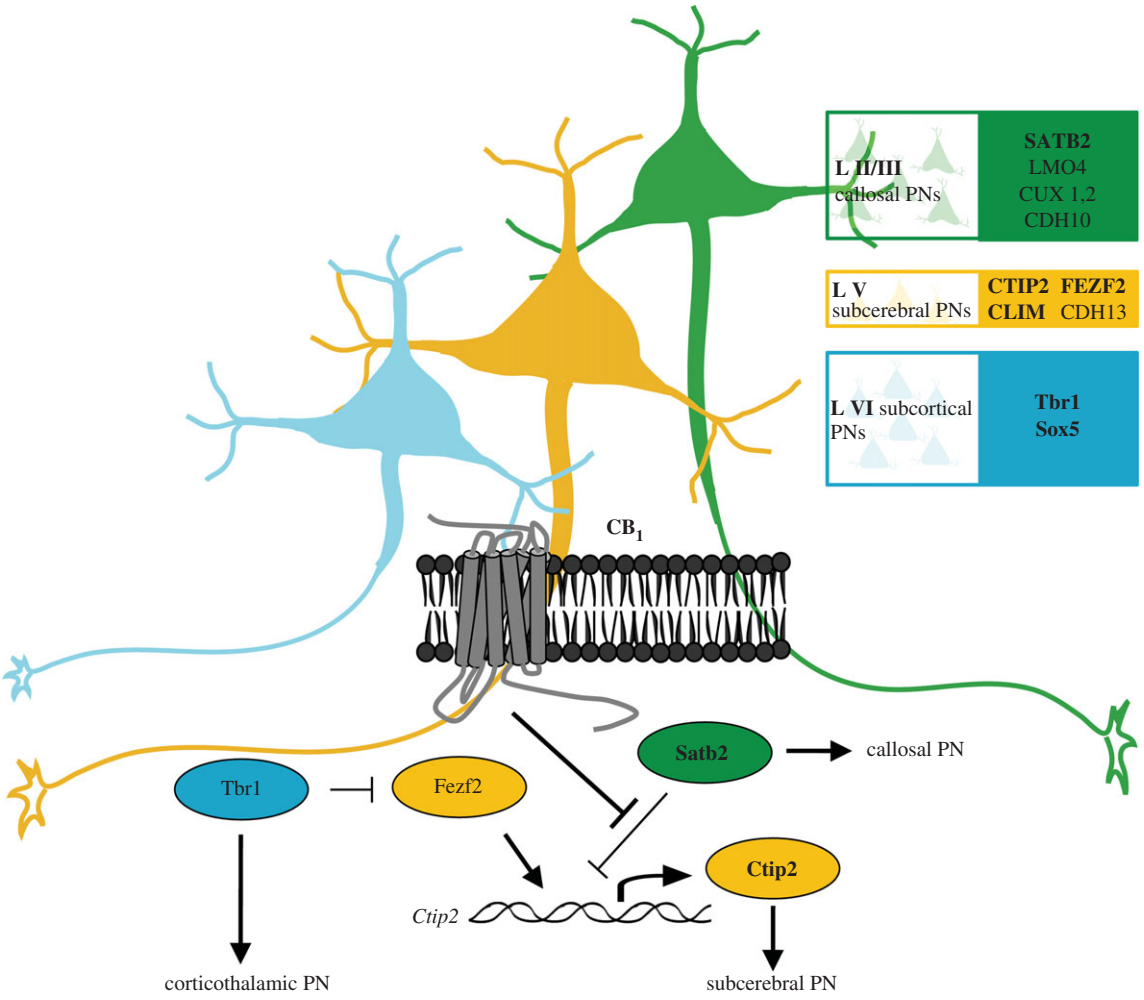

Figure 4. CB_1 cannabinoid receptor signalling and neuronal differentiation. CB_1 receptor activity in differentiating cortical neurons is coupled by as yet unknown mechanisms to the modulation of the neurogenic transcription factor code Ctip2-Satb2. CB_1 receptors are positively coupled to COUP-TF II interacting protein 2 (Ctip2) and negatively to Satb2-mediated repression of Ctip2. Thus, CB_1 receptor activity tunes the transcriptional neurogenic programme responsible for upper and lower cortical neuron differentiation. Transcription factors involved in cortical laminar specification regulated by CB_1 receptor are indicated in bold letters.

(a) *CB_1 cannabinoid receptor-mediated regulation of gene expression*

CB_1 receptor activation can regulate more than 20 transcription factors that are part of the gene expression signatures involved in NP maintenance, neuronal commitment and maturation [81]. CB_1 receptor signalling converges onto the activation of STAT3, a transcription factor responsible for gene expression regulation that is involved in cannabinoid-induced neurite outgrowth and ERK activation [76]. In neuroblastoma cells, CB_1 receptor-induced STAT3 activation relies on PI3K-dependent activation of the transcription factor Pax6 [81], a paired box family member essential for the generation of glutamatergic neurons and cortical neurogenesis [82]. In addition, CB_1 receptor prevents the inhibitory effect of breast cancer resistance associated on neuritogenesis [81]. During cortical development and pyramidal neurogenesis, CB_1 receptors are also able to modulate Pax6 and Tbr2 transcriptional activity in VZ/SVZ progenitors (Díaz-Alonso *et al.* 2012, unpublished results). Noteworthy, chronic administration of a Δ^9-tetrahydrocannabinol analogue severely disrupted chick neural development, and this was associated to gene expression changes of critical neurogenic transcription factors, including Krox20, Otx2, Pax6 and Sox2 [11]. Unfortunately, the involvement of the CB_1 receptor in these actions was not investigated. CB_1 receptor activity in differentiating cortical neurons is coupled by as yet unknown mechanisms [83] to the modulation of the neurogenic transcription factor code Ctip2-Satb2 (figure 4) [84]. CB_1 receptors are positively coupled to COUP-TF II interacting protein 2 (Ctip2) and negatively to Satb2-mediated repression of Ctip2. Thus, CB_1 receptor activity tunes the transcriptional neurogenic programme responsible for upper and lower cortical neuron differentiation, and CB_1 receptor inactivation results in reduced $Ctip2^+$ corticospinal projection neuron development that affects in turn motor function in adulthood [83].

The involvement of the CB_1 receptor in embryonic neuronal development [85], but also in postnatal astrogliogenesis [27] and oligodendrocyte survival and myelination [80,86], suggests that CB_1 receptor signalling could also target still unknown pro-gliogenic transcription factors [3]. ECB signalling may be involved in tumour-initiating stem cell decisions of

proliferation versus cell cycle exit and differentiation [87], and CB_1 receptor regulation of STAT3 is a likely candidate to mediate CBI regulation of astrogliogenesis [88]. In summary, the CB_1 receptor exerts a dual role, pro-neurogenic in some cases and progliogenic in others, thus indicating that differences in the intrinsic progenitor features and/or in the surrounding niche may be responsible for alternative CB_1 receptor-driven neurogenic outcomes.

6. PATHOPHYSIOLOGICAL IMPLICATIONS OF THE NEURODEVELOPMENTAL ROLE OF CB_1 CANNABINOID RECEPTORS

The neurodevelopmental role of the ECB system reveals that altered cannabinoid signalling, due to either hyper- or hypo-function of the CB_1 receptor, can exert long-lasting consequences in adult brain neuronal function by modifying the actively developing brain. Neurodevelopmental disorders can originate by subtle or severe alterations of various neurogenic processes, including neuronal generation, migration, maturation and connectivity that are responsible for adult brain dysfunction [89]. Among developmental disorders, cortical alterations constitute an important example of how embryonic deficits affect adult neurological function. As previously discussed, CB_1 receptor signalling plays a regulatory role in different neural cell fate processes involved in these pathologies. Genetic polymorphisms of cannabinoid receptors can induce subtle changes during development by influencing signalling strength or duration and later, when synaptic transmission ensues, by influencing the appropriate balance of neuronal activity. Likewise, mutations of ECB-metabolizing enzymes, including degrading (FAAH, ABHD6/12, MGL) or synthesizing enzymes (NAPE-PLD, DAGL), may result in less active enzymes that would increase or reduce ECB tone and signalling. In this regard, FAAH polymorphisms have been associated with drug abuse behaviours [90,91]. A recent proof of concept of this notion is the involvement of ABDH12 mutations that associate with the neurodegenerative disease polyneuropathy, hearing loss, ataxia, retinitis pigmentosa and cataract (PHARC) that occurs with concomitant demyelination and cerebellar ataxia [92].

CB_1 receptor signalling can be influenced as well by prenatal exposure to marijuana-derived cannabinoids or by contact with drugs targeting either directly or indirectly the ECB system. The neurobiological consequences of plant-derived cannabinoid intake on pre- and postnatal stages have been recently reviewed from the perspective of animal models and humans [6,93], and indicate that the brain burst period is of especial susceptibility. According to the developmental stage in which CB_1 receptor signalling is functional, its interference may affect different neural cell populations, including neuronal generation and specification (embryonic stages) [13,37], glial development (postnatal stages) [27,80] and neuronal maturation and connectivity [13,32,94]. Blockade of the CB_1 receptor when the neurogenic wave responsible for deep cortical neuronal generation is active affects corticospinal neuronal specification, thereby tuning subcerebral-versus callosal neuron-projections and thus skilled motor function in adulthood [83]. In addition, CB_1 receptor expression, first in white matter and later in postnatal grey matter, participates in whisker barrel map development of the somatosensory cortex, supporting the contribution of the CB_1 receptor for the appropriate integration of sensory information input [95]. In summary, the regulatory role of the CB_1 receptor in cortical development processes has the potential to exert significant impact on adult brain function [96,97].

Developmental interference of cannabinoid signalling can influence human emotion-, threat- and reward-related brain function at different levels [6,26]. Polymorphisms of the *CNR1* gene, which encodes the CB_1 receptor, may reduce or enhance G-protein-mediated signalling and have been associated to major depression, psychoses and schizophrenia [98,99]. Unexpectedly, polymorphisms of the CB_2 receptor-encoding gene, *CNR2*, may associate with depressive syndromes and schizophrenia [100]. Changes in the appropriate number, specification or migration of projection neurons and interneurons will result in modifications of neuronal activity that in turn will be followed by a more generalized neurochemical unbalance. The glutamatergic neuronal dysfunction hypothesis of schizophrenia [101] suggests that malfunction of the CB_1 receptors in pyramidal neurogenesis may contribute to the pathogenesis of psychoses or schizophrenia symptoms. Malfunction of the ECB system may be one of the causes underlying neuronal dysfunction, but alternatively the CB_1 receptor and ECB-metabolizing enzymes are also likely to adapt to aberrant neuronal homeostasis as an attempt to counteract the changes of neuronal transmission [102]. Thus, cortical glutamic acid decarboxylase 67 deficiency, a typical neurochemical marker of schizophrenia, results in lower CB_1 receptor expression. It remains unknown whether these kind of ECB system adaptations exert positive effects to cope with those alterations, or worsen the pathological processes.

(a) *Neurodevelopmental disorders: epileptogenesis*

One of the most common consequences of cortical development alterations is the appearance of epileptic foci due to alterations in neuronal excitability [89,103]. Considering the dual role of the CB_1 receptor in the generation and maturation of excitatory and inhibitory neurons it can be predicted that CB_1 receptor-dependent signalling alterations during development would impact the appropriate excitation/inhibition balance of the mature brain. Ablation of the CB_1 receptor interferes with cortical progenitor proliferation [29], the correct specification of upper/lower cortical neurons [83] and axonal growth and fasciculation [13,32]. Thus, unbalanced CB_1 receptor activity and its consequences in cortical pyramidal neurogenesis may elicit epileptic syndromes similar to those associated with cortical dysplasia, tuberous sclerosis or heterotopias [89]. Deletion of doublecortin, a microtubule-associated protein characteristic of migrating neuroblasts that is responsible for lissencephaly,

interferes with excitatory neuron radial migration [104], induces lamination alterations and has a profound impact on neuronal excitability [105]. These findings suggest that exacerbated excitotoxicity in CB_1-deficient mice [68] and the involvement of the ECB system in seizure threshold and epilepsy [106,107] may, at least in part, be due to developmental cortical alterations that result in unbalanced excitation/inhibition activity.

In addition to excitatory neuronal alterations, unbalanced generation of interneuron populations contribute to developmental epilepsies [103]. As the ECB system is involved in the development and morphogenesis of inhibitory neurons [15,20], it is likely that these developmental alterations may be responsible for changes in the susceptibility to epileptogenesis. Disruption of cortical interneuron development is known to exert GABAergic cell type-specific deficits, epilepsy and behavioural dysfunction [108,109]. Thus, the decrease in the number of interneurons and disruption of appropriate inhibitory synapse development observed in Dlx1-deficient mice, a homeodomain transcription factor essential during embryonic development for the production of forebrain GABAergic interneurons, is associated with a reduction of GABA-mediated inhibitory postsynaptic currents, electrographic seizures and cortical dysrhythmia *in vivo* [109]. Ablation of neurogenic transcription factors during development interferes with cortical excitation/inhibition balance and, for example, COUP-TFI knockout mice display altered balance of the development of medial versus caudal ganglionic eminence interneurons [110]. Whether the CB_1 receptor plays a role in the differentiation and development of the different interneuron populations is still unknown. However, defective CB_1 receptor function in $CCK^+vGlut3^+$ basket neuron development would conceivably affect the excitation/inhibition balance by interfering with interneuron-mediated inhibition. In agreement with this notion, experimental models of epilepsy result in predominant loss of $CCK^+CB_1^+$ basket interneurons [111], and indiscriminate loss of local-circuit hippocampal interneurons triggers network hyperexcitability, loss of CA1 pyramidal cells and hippocampal epileptiform seizures [112]. Chronic cannabinoid administration induces alterations of CCK^+ interneuron density in the hippocampus and cortex [15,20] that are likely to interfere with the balance of inhibition/excitation and thus may result in the development of epileptogenic foci.

Once neuronal activity is established, the absence or interference with CB_1-mediated neuromodulation would constitute a major mechanism for unbalanced neuronal activity through the disruption of excitatory and inhibitory activity [8,22]. CB_1 receptor engagement by retrograde ECB messengers is a key regulator of synaptic plasticity, both of inhibitory synapses (depolarization-induced suppression of inhibition and long-term depression of inhibitory transmission) and excitatory synapses (depolarization-induced suppression of excitation and long-term depression of excitatory transmission) [8,22,113]. Thus, CB_1 receptor blockade induces epileptic discharges that have been attributed to the absence of depolarization-induced suppression of GABA postsynaptic currents [114]. CB_1 receptors are involved in limbic hyperexcitability and fever-induced seizures through the potentiation of depolarization-induced suppression of inhibition in CCK^+ interneurons [105,115]. In addition, CB_1 receptors expressed solely in excitatory hippocampal vGlut1 neurons can allow protection from kainic acid-induced seizures [18,68]. It is important to note that, as within the early stages of brain development GABA is excitatory instead of inhibitory, CB_1 receptor activation and subsequent inhibition of GABA release would result in different outcomes depending on the developmental stage in which the ECB system function is altered.

7. CONCLUSIONS

Developmental neurobiology studies have started to elucidate the contribution of CB_1 receptor signalling in appropriate nervous system formation. These studies have underscored the active role of ECBs as local cues of neurogenic niches that, via the CB_1 receptor, drive progenitor cell proliferation/cell cycle progression, control neuronal migration and tune neuronal differentiation/specification. At early developmental stages, the CB_1 receptor and a precisely regulated ECB tone act as signalling cues in neurogenic niches [44,84]. CB_1 receptor activity exerts a critical regulatory role in different neural cell fate decisions, i.e. (i) cell cycle progression and proliferation; (ii) neural cell specification; and (iii) migration and morphogenesis. Dysfunction of the ECB system may be a determinant of seizure onset and epileptogenesis as a consequence of unbalanced excitatory and inhibitory neurotransmission [116,117]. At postnatal stages, acute or long-lasting CB_1 receptor-mediated neuromodulation upon cannabinoid exposure or altered ECB signalling interferes with neuronal maturation and tunes neuronal connectivity and developing circuits, which may in turn exert relevant consequences on adult neuronal function [6]. In summary, the CB_1 receptor exerts a key regulatory role in cortical developmental and this may have significant consequences in adult brain function, including the tuning of an appropriate balance of neuronal excitation/inhibition activity and the susceptibility to suffer neuropsychiatric disorders.

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Review

Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation

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ABSTRACT

Cannabinoids, the active components of cannabis (*Cannabis sativa*) extracts, have attracted the attention of human civilizations for centuries, much earlier than the discovery and characterization of their substrate of action, the endocannabinoid system (ECS). The latter is an ensemble of endogenous lipids, their receptors [in particular type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors] and metabolic enzymes. Cannabinoid signaling regulates cell proliferation, differentiation and survival, with different outcomes depending on the molecular targets and cellular context involved. Cannabinoid receptors are expressed and functional from the very early developmental stages, when they regulate embryonic and trophoblast stem cell survival and differentiation, and thus may affect the formation of manifold adult specialized tissues derived from the three different germ layers (ectoderm, mesoderm and endoderm). In the ectoderm-derived nervous system, both CB_1 and CB_2 receptors are present in neural progenitor/stem cells and control their self-renewal, proliferation and differentiation. CB_1 and CB_2 show opposite patterns of expression, the former increasing and the latter decreasing along neuronal differentiation. Recently, endocannabinoid (eCB) signaling has also been shown to regulate proliferation and differentiation of mesoderm-derived hematopoietic and mesenchymal stem cells, with a key role in determining the formation of several cell types in peripheral tissues, including blood cells, adipocytes, osteoblasts/osteoclasts and epithelial cells. Here, we will review these new findings, which unveil the involvement of eCB signaling in the regulation of progenitor/stem cell fate in the nervous system and in the periphery. The developmental regulation of cannabinoid receptor expression and cellular/subcellular localization, together with their role in progenitor/stem cell biology, may have important implications in human health and disease.

Contents

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonoylethanolamine; BDNF, brain derived neurotrophic factor; CBD, cannabidiol; CBG, cannabigerol; CFU-GEMM, colony-forming unit: granulocyte, erythrocyte, macrophage, megakaryocyte; CREB, cAMP response element-binding protein; CSF, colony-stimulating factors; DAGL, diacylglycerol lipase; ECB, endocannabinoid; ERK, extracellular-signaling regulated protein kinase; ES, embryonic stem; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; FGF, fibroblast growth factor; GAD, glutamate decarboxylase; GSK3β, glycogen synthase kina; ICM, inner cell mass; HSC, hematopoietic stem cells; HPC, hematopoietic progenitor cells; L1-CAM, L1-cell adhesion molecule; MAGL, monoacylglycerol lipase; mGluR, metabotropic glutamate receptors; mTORC1, mammalian target of rapamycin complex 1; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; NP, neural progenitor/stem cell; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; PI3K, phosphoinositol 3-kinase; PKA, protein kinase-A; PPARγ, peroxisome proliferator activated receptors; RANKL, receptor activator of nuclear factor kappa-B ligand; SVZ, subventricular zone; THC, Δ^9-tetrahydrocannabinol; vGlut, vesicular glutamate transporter; VZ, ventricular zone.

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1. The endocannabinoid system at a glance

1.1. Plant-derived and endogenous cannabinoids

The plant-derived cannabinoids (phytocannabinoids) are more than 80 lipid-soluble compounds found in the resin produced by female plants of the cannabis (*Cannabis sativa*) herb, and are synthesized from fatty acid precursors via a series of transferases and synthases [1,2]. Since ancient times the recreational use of cannabis has been known, and was first described by Dioscorides already in 60 A.D. Yet, it was only in the 19th century that its medical use was introduced in UK for its analgesic, anti-inflammatory, anti-emetic and anti-convulsing properties, and remained in use until prohibition in the early 20th century. All phytocannabinoids are uniquely found in cannabis and the two major substances, Δ^9-tetrahydrocannabinol (THC, the main psychoactive ingredient), and cannabidiol (CBD) are derived from the common synthetic precursor cannabigerol (CBG).

THC was isolated, identified and synthesized ~50 years ago [3], but it was only ~30 years later that cannabinoid receptors were described and cloned in the brain, explaining the mode of action of cannabis extracts and leading to identification and isolation of their endogenous counterparts [4]. Among the latter compounds, amides, esters, and ethers of long chain polyunsaturated fatty acids are included and these are collectively termed "endocannabinoids" (eCBs). The first discovered and best-characterized eCBs are *N*-arachidonoylethanolamine (also known as anandamide, AEA) and 2-arachidonoylglycerol (2-AG) [5]. Some other compounds have been proposed as members of the eCBs family, including 2-AG-ether (noladin ether), *O*-arachidonoylethanolamine (virodhamine, an "inverted" AEA), and relevant eCB-like compounds such as *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA) [6,7]. Structures of the main phytocannabinoids, eCBs and eCB-like compounds are shown in Fig. 1.

1.2. Metabolism and target receptors of endocannabinoids

The eCBs, together with their molecular targets and metabolic enzymes, form the so-called "endocannabinoid system" (ECS), schematically depicted in Fig. 2. eCBs are synthesized and released in response to physiological or pathological stimuli. AEA biosynthesis includes two steps: *N*-arachidonoylphosphatidylethanolamine (NArPE) is formed from phosphatidylethanolamine by a calcium-dependent *N*-acyltransferase, and then it is converted through at least five distinct metabolic pathways into AEA. The most studied route for such a conversion involves the *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) [8], but other relevant pathways engage PLA and *lyso*-PLD [9], α/β-hydrolase 4 and glycerophosphodiesterase 1 [10,11], or PLC and protein tyrosine phosphatase type-22 [12]. The biosynthesis of 2-AG starts from *sn*-1-acyl-2-arachidonoylglycerols (DAGs), that can be directly converted into 2-AG through the action of two Ca^{2+}-sensitive *sn*-2-selective DAG lipases, i.e. DAGL-α and DAGL-β [13].

Once synthesized, eCBs bind to and functionally activate their target receptors, causing several biological effects on different tissues [14]. The main receptor targets for both phytocannabinoids and eCBs are type-1 (CB_1) and type-2 (CB_2) G protein-coupled cannabinoid receptors [15,16]. CB_1 is widely expressed in the nervous system mainly at the terminal ends of central and peripheral neurons, and its presence has also been widely investigated at many different extra-neural sites [14]. Once activated, CB_1 is involved in the inhibition of excitatory and inhibitory neurotransmission and can modulate cognitive, memory and motor functions, as well as analgesia [17–19]. CB_2 is mainly expressed in the cells of the immune system where it is commonly associated with the regulation of different immune functions [20–23]. The identification of CB_2 in brainstem neurons [2,24] and its presence in activated microglial cells and astrocytes [25,26], or in certain subsets of neurons upon insult [27], has led to an "identity crisis" of this receptor [28]. Indeed, the activation of CB_2 is associated with chronic inflammation of the nervous system, as well as with several immunological, cardiovascular and bone disorders [29–33].

Other targets of AEA include the transient receptor potential (TRP) superfamily of cation channels [34,35], in particular vanilloid receptor-1 (TRPV-1) [36], which is expressed in sensory neurons and in epithelial, endothelial and immune cells [14,37]. Recently, the peroxisome proliferator activated receptors (PPARs), a family of nuclear receptors able to alter lipid turnover and metabolism, have been shown to weakly bind AEA and 2-AG, as well as some plant-derived and synthetic cannabinoids [38].

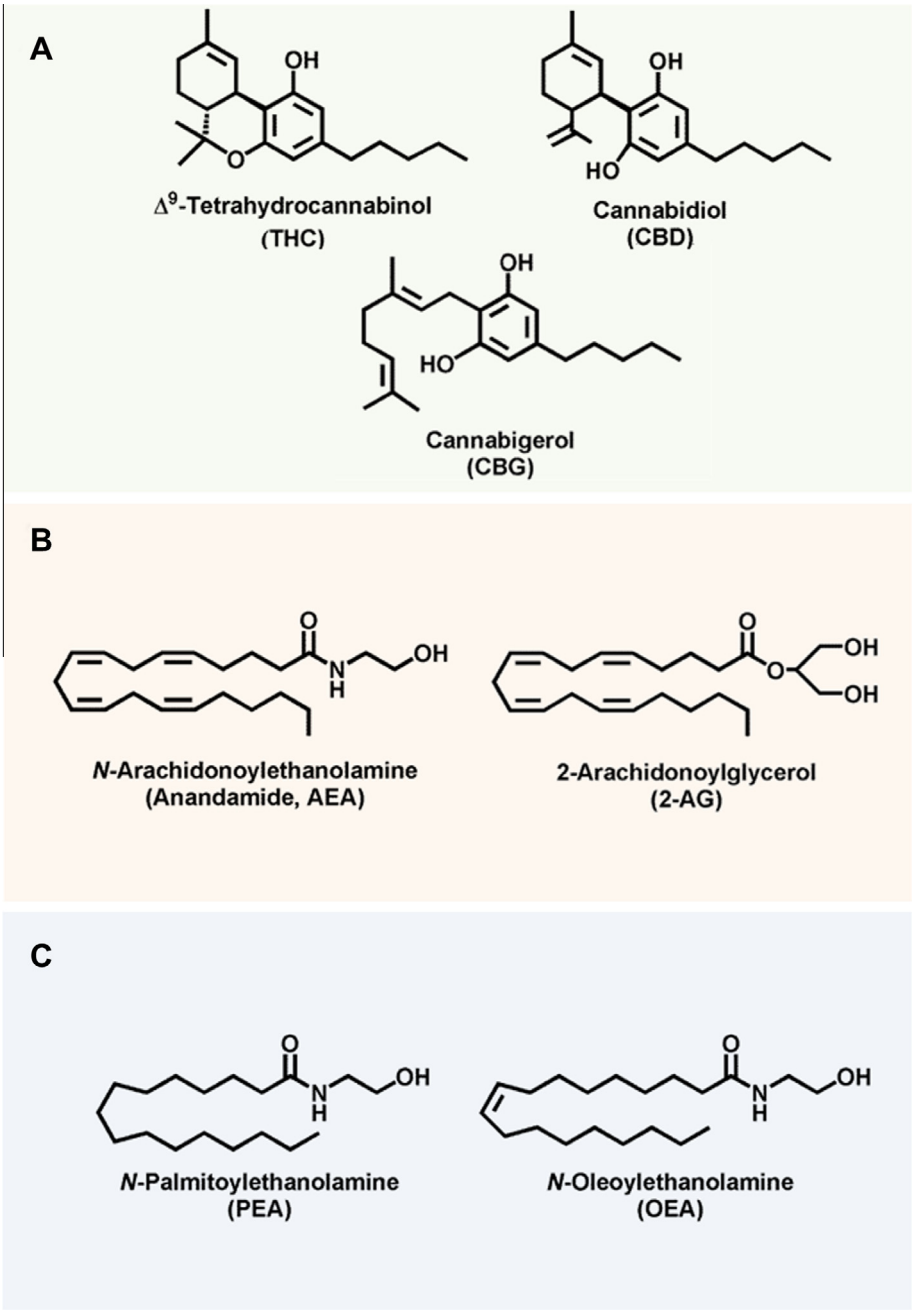

Fig. 1. Chemical structures of the main plant-derived (A) and endogenous cannabinoids (B), as well as of endocannabinoid-like compounds (C).

AEA and 2-AG are then inactivated by a two-step process: cellular uptake through a purported "endocannabinoid membrane transporter" (EMT) [39], and intracellular hydrolysis. AEA is principally cleaved by fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine [40,41], but also another enzyme, *N*-acylethanolamine acid amidase (NAAA), is involved in its hydrolysis, with as yet unclear physiological implications [42]. 2-AG can be cleaved into glycerol and arachidonic acid by FAAH, though its main hydrolase is a monoacylglycerol lipase (MAGL), responsible for ~85% of 2-AG hydrolysis in the mouse brain [43]. In addition, 2-AG can also be cleaved by two integral membrane proteins, α/β-hydrolase domain-containing protein 6 (ABHD6) and 12 (ABHD12) [44,45]. Furthermore, AEA and 2-AG are substrates of cyclooxygenase-2 (COX-2), different lipoxygenase (LOX) isozymes and cytochrome P450, leading to oxidized compounds like prostaglandin-ethanolamides and -glyceryl esters, hydroxy-anandamides and hydroxyeicosatetraenoyl-glycerols, respectively, all endowed with distinct biological activities [46].

1.3. Main signaling pathways triggered by cannabinoid receptors

CB_1 and CB_2 are metabotropic receptors that usually couple to heterotrimeric $G_{i/o}$ proteins, and thus trigger the canonical signaling pathway of inhibition of adenylyl cyclase activity and reduction of cAMP levels [47]. In some circumstances, e.g. according to the availability of G protein subunits, dimerization with other G-protein-coupled receptors or particular cell environments, different coupling partners such as $G_{q/11}$ or G_s may occur, and therefore exert different consequences in signaling regulation [14]. In the nervous system, fast and short-term CB_1-mediated inhibition of

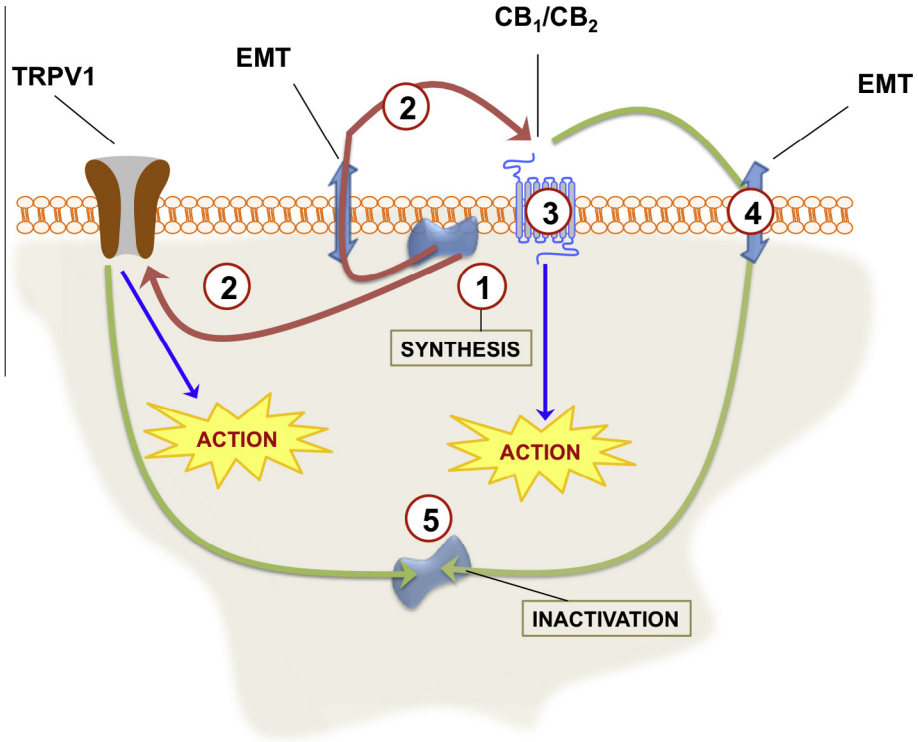

Fig. 2. Metabolism of eCBs. Red and green arrows represent the activation and inactivation steps, respectively. 1-Synthesis. AEA and 2-AG are released on demand from membrane lipids, through the activity of NAPE-PLD and DAGL, respectively; 2-Transport. AEA and 2-AG move across the plasma membrane via a purported EMT; 3-Action. Targets of AEA and 2-AG are CB_1 and CB_2, that show an extracellular binding site. AEA also binds to type-1 vanilloid receptor (TRPV1) that bears an intracellular binding site. Once eCBs bind to their target receptors, different signaling pathways can be activated depending on the cellular environment; 4. Reuptake. After their actions, eCBs are taken up by EMT for inactivation; 5-Inactivation. AEA is hydrolyzed by FAAH to ethanolamine and arachidonic acid, whereas 2-AG is hydrolyzed by MAGL and to a minor extent by FAAH, that releases glycerol and arachidonic acid. Moreover, AEA and 2-AG are also oxidized by cyclooxygenase-2, lipoxygenases and cytochrome P450, to produce prostaglandins and hydroxyl-derivatives (omitted for the sake of clarity). See text for details.

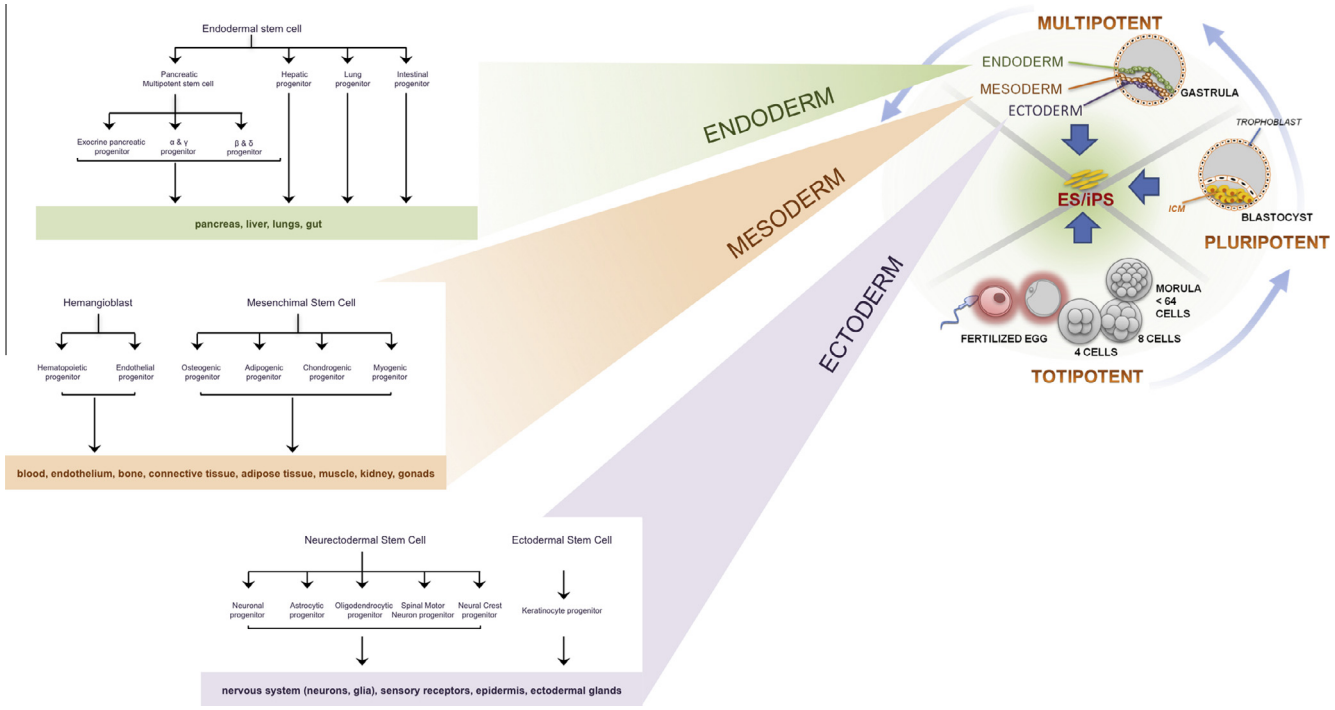

Fig. 3. Origin, classification and fate of stem cells. See text for details.

neurotransmitter release relies on $G_{\beta\gamma}$ subunit-evoked voltage-sensitive Ca^{2+} channel inhibition. In addition, long-term depression involves reduced protein kinase A activity and increased Ca^{2+}-dependent activity of the protein phosphatase calcineurin, that finally controls neurotransmitter release by regulating synaptic vesicle dynamics via Rab3-interacting molecule-1α. CB_2

receptor signaling is assumed to be largely overlapping with CB_1 receptor signaling, with the obvious difference, however, of their distinct cell-type distribution [28]. CB_1 and CB_2 regulate various protein kinase cascades involved in cell proliferation and survival, with major consequences on progenitor cell fate decisions. Thus, both cannabinoid receptors regulate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) and extracellular-signaling regulated protein kinase (ERK) pathways in most of the cell systems investigated. A detailed discussion of the signaling mechanisms involved in neural progenitor/stem cell fate regulation by CB_1/CB_2 receptors can be found in Section 3.

2. Progenitor/stem cells and early embryogenesis

2.1. Stem cells at a glance

The field of stem cell biology has rapidly expanded since the identification of hematopoietic stem cells (HSC) back in 1963 by Till and McCullough [48]. Stem cells are undifferentiated cells that have the potential to develop into many differentiated cell types. In mammals, there are two main types of stem cells: embryonic stem (ES) cells, which are isolated from the inner cell mass (ICM) of blastocysts, and adult stem cells, which are found in various tissues [49,50]. By definition, a stem cell requires the property of self-renewal, namely the ability to go through numerous cycles of cell division while maintaining the undifferentiated state, and the property of cell potential, which defines the ability to originate many different specialized cell types. During embryogenesis only the zygote is "totipotent", because it has the ability to divide and produce an entire organism, including extraembryonic tissues. Instead, ES cells are considered "pluripotent", because they can differentiate in any of the three germ layers (i.e., endoderm, mesoderm and ectoderm), each of which further divides into progenitor cells that are "multipotent", having the potential to give rise to cells from multiple (but limited in number) lineages (Fig. 3) [49,50]. Till recently there was a general consensus that only some tissues had the ability to renew themselves, but now it is clear that almost every adult tissue has a small percentage of progenitor/stem cells, designed to replace damaged cells in the same tissue, and thus acting as a repair system for the body [51]. A significant breakthrough in understanding stem cell biology was achieved by Yamanaka and co-workers, who demonstrated the "reprogramming" of adult human fibroblasts into induced pluripotent stem cells through the retroviral-mediated introduction of a discrete subset of transcriptional regulators [52]. Remarkably, the stem cells generated with this technology have proven to be similar to ES cells, opening up interesting perspectives for future therapeutic approaches of many human diseases. Understanding the molecular mechanisms responsible for stem/progenitor cell generation and differentiation is therefore not only a key question in developmental biology, but also a pre-requisite for the potential application of progenitor/stem cells in therapeutic grounds. A significant part of research efforts includes the possibility to clearly identify distinct stem cell lineages. A brief classification of the different progenitor/stem cells originating from the three germ layers is schematically represented in Fig. 3. Here, we will focus mainly on neural progenitor/stem cells, hematopoietic stem cells, and mesenchymal stem cells, in which the role of cannabinoid signaling has been investigated in closer detail.

2.2. (Endo)cannabinoid regulation of early embryogenesis

In the early stages of development, a functional ECS has been reported in both ES cells and trophoblast stem cells [53–59]. Interestingly, several studies demonstrated that both phytocannabinoids and eCBs arrest the development of early embryos into blastocysts, mainly through a CB_1-dependent mechanism [60–63]. However, both cannabinoid receptors and their endogenous ligands were significantly induced during the formation of ES-derived embryoid bodies, and pharmacological blockade of CB_1 and CB_2 was shown to induce ES cell death, suggesting that eCBs are involved in the survival of ES cells [56]. Furthermore, the presence of FAAH mRNA in early embryos seems to reflect the accumulation of maternal message and its presence in blastocysts indicates embryonic gene activation, thus providing evidence that FAAH modulates local levels of AEA that could control embryo development [53]. Up to date, the role of CB_2 in ES cells and early embryos remains poorly understood, and it is likely to contribute to the development of ICM into the fetus. It is also conceivable that CB_2 plays a role in specifying pluripotent ICM cell lineage during blastocyst formation [56]. Activation of both cannabinoid receptors in differentiating embryoid bodies does not lead to increased cell proliferation, but rather reduces the percentage of cells undergoing cell death [63]. On this basis, it was suggested that both CB_1 and CB_2 are involved in ES cell survival, possibly because they trigger an autophagic pathway in early embryos when eCBs are present at high concentrations [64]. Of note, eCBs do not lead to an overt alteration of the expression of genes that are early markers of ES cells or late markers of lineage differentiation into embryoid bodies, leaving open the question of the actual role of eCBs in tuning "stemness" and differentiation potential of ES [59].

The eCB signaling has been shown to exert a critical role also in controlling trophoblast proliferation and differentiation. Indeed, AEA affects trophoblast outgrowth through the regulation of the fibronectin-binding activity of blastocysts, which is decreased by high AEA levels that influence trophoblast invasion [61]. Additionally, embryos exposed to high levels of AEA showed reduced trophoblast proliferation and subsequent implantation failure mainly via CB_2 [58,65], and an aberrant eCB signaling leads to premature trophoblast stem cell differentiation [66]. These data are also corroborated by the evidence that doses of THC equivalent to those found in the serum of cannabis users inhibit proliferation of trophoblast stem cells, by activating several genes that encode for growth, apoptosis, cell morphology and ion exchange pathways controllers [67].

Overall, the current notion is that, at early embryonic stages, CB_1 plays a fundamental role in the surveillance of normal growth of the embryo, whereas CB_2 may be responsible for controlling stem cell populations [68]. Understanding how these two receptor types finely coordinate their actions during embryogenesis may shed light on their specific functions and on their possible pharmacological exploitation to promote ES/trophoblast cell differentiation (See Fig. 4).

3. The endocannabinoid system in neural progenitor/stem cells

In the developing nervous system and the restricted neurogenic areas that persist in the adult brain (the hippocampal subgranular zone and subventricular zone) the ECS exerts a regulatory role on neural progenitor/stem (NP) cell proliferation, differentiation and migration by engaging CB_1 receptors [69,70]. CB_1 receptors are expressed, albeit at low levels, in neuroepithelial progenitor cells from early embryonic stages, and their levels increase along neural differentiation. In embryonic stages CB_1 is enriched in white matter areas, until the acquisition of its final expression pattern in the adult nervous system [71,72]. In addition CB_2 receptors, which are normally absent in neurons, are functionally active in undifferentiated neural cells and may participate, together with CB_1 receptors, in the regulation of NP cell fate decisions including cell

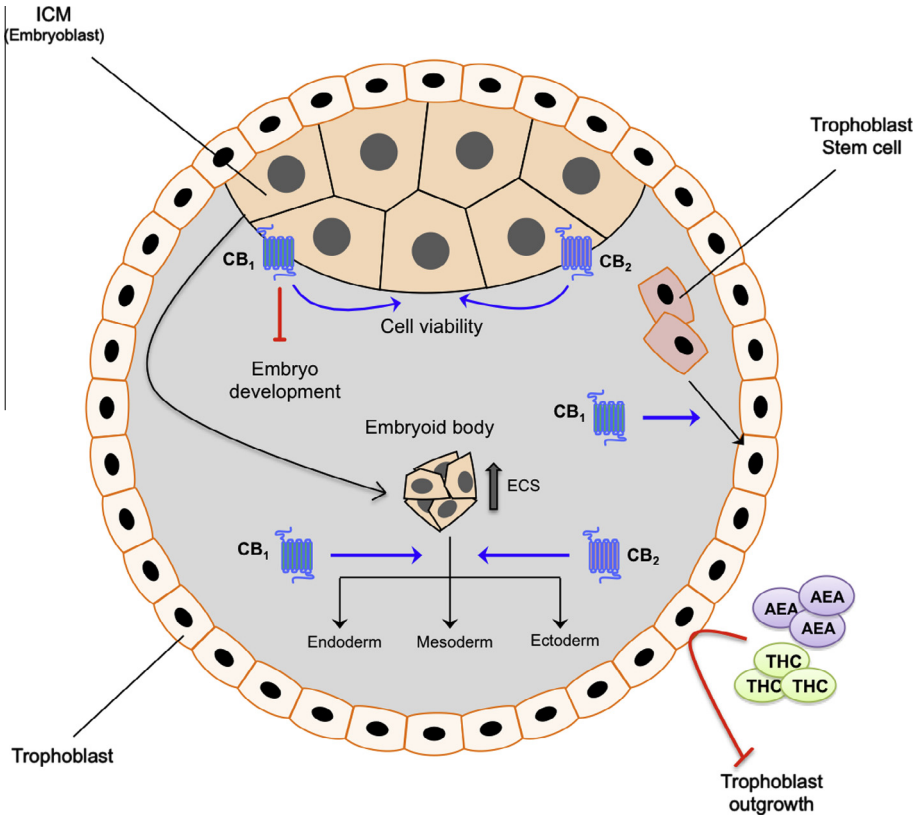

Fig. 4. Cannabinoid signaling in the blastocyst. The blastocyst is composed of an inner cell mass (embryoblast), which is the source of embryonic stem cells and subsequently forms the embryo, and an outer layer of cells, the trophoblast, which later on forms the placenta. Pluripotent embryonic stem cells form embryonic stem cell aggregates (termed embryoid bodies), which contain a number of different cell types that give rise to all three germ layers of the developing embryo (endoderm, mesoderm and ectoderm). Early disruption of the pluripotent network of embryonic stem cells generates a population of trophoblast stem cells, which provide the precursors of the trophoblast. eCBs influence proliferation and differentiation of both embryonic stem cells and trophoblast stem cells at multiple levels. See text for details and abbreviations.

proliferation, cell cycle maintenance and neural differentiation [73–75].

3.1. Cannabinoid receptor expression in neural progenitors and the endocannabinoid tone in neurogenic niches

In neural cells CB_1 and CB_2 receptors show opposite patterns of expression, with CB_1 increasing and CB_2 decreasing along neuronal differentiation (Fig. 5A) [73,76–78]. During neuronal differentiation, CB_1 expression is induced by neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), but the signaling mechanisms downstream of TrkB and TrkA receptors that control CB_1 activity remain largely unexplored [79,80]. Increased CB_1 expression along excitatory and inhibitory neuronal lineage progression allows eCBs to control neuronal specification and morphogenesis [70,76,81–83]. Although the ability of CB_1 signaling to act as molecular switch of neurochemical specification is unknown, CB_1 receptor levels are associated to increased expression of differentiation markers of various neuronal lineages. CB_1 activity increased the number of pyramidal vesicular Glu transporter 1^+ (vGLUT1) neurites [82], vesicular GABA transporter 1^+/vGLUT3$^+$ interneuron inputs [81], and choline acetyltransferase$^+$ cholinergic differentiation [80]. In mature GABAergic interneurons CB_1 levels are controlled by the expression of the GABA-synthesizing enzyme glutamate decarboxylase, 67-kDa isoform (GAD67) [84], and CB_1 receptor expression in striatal neurons is controlled by the transcription factor REST via RE1 sites [85].

Cannabinoid receptors present in NPs at neurogenic niches are engaged by eCBs conceivably owing to 2-AG and AEA synthesis by surrounding neurons [72] and, in a paracrine/autocrine manner, by NPs themselves [86] and ependymal cells [87]. Whereas in the mature nervous system neuronal activity engages on-demand eCB generation, that in turn exerts a neuromodulatory role in differentiated neurons [47], the extracellular or intrinsic mechanisms responsible for eCB production in active neurogenic niches are not fully understood. NPs produce and release the two major eCB species, namely AEA and 2-AG, in response to increased intracellular Ca^{2+} concentration (Fig. 5B) [86] and the eCB tone contributes to basal and stimulus-induced NP proliferation via CB_1 receptors [88–90]. In addition, it has been shown that 2-AG is present in neurogenic niches and plays an active role in NP cell regulation owing to the dynamic tuning of 2-AG levels by DAGL and MAGL activity [91]. Genetic ablation of DAGLα, but not of the β isoform, interferes with hippocampal and SVZ-derived neurogenesis [92], and pharmacological inhibition of DAGL-activity in NP cultures reduces cell proliferation [74].

Regarding the extracellular signaling cues known to mobilize eCBs, fibroblast growth factor (FGF) in coordination with neural cell adhesion molecule (NCAM) increases 2-AG levels via DAGL coupled with PLCγ activation (Fig. 5B) [93]. NGF via TrkA enhances 2-AG production during neurite outgrowth of cholinergic neurons by upregulating the expression of ECS proteins [80]. High expression levels of DAGLα in NSCs have been shown to rapidly decrease along their differentiation into GABAergic neuronal cells [94], through a mechanism that relies on the regulation of the transcriptional regulator Specificity protein 1. In neuroblastoma cells retinoic acid-induced neuronal-like differentiation increases first DAGLα expression and later DAGLβ [95]. Less is known on the mechanism of AEA generation in NPs, and the expression pattern of NAPE-PLD and ABD enzymes remain unknown. NPs express FAAH, the major enzyme involved in AEA degradation, and its genetic ablation or pharmacological inhibition increases AEA levels

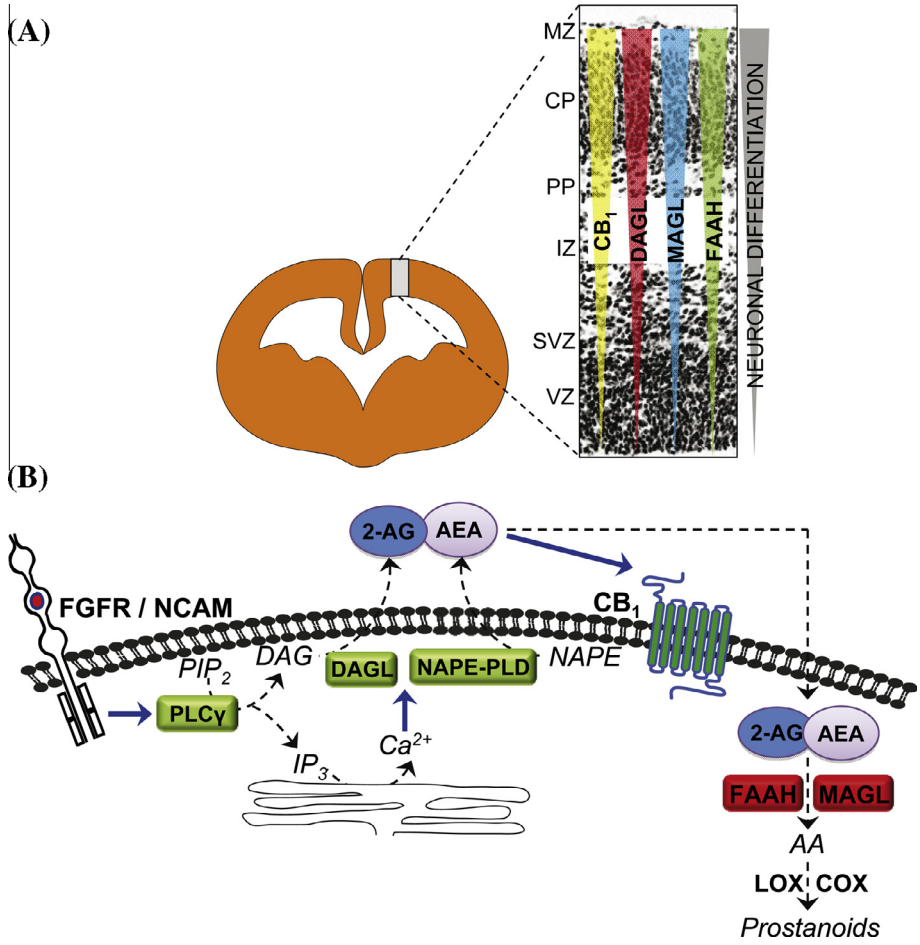

Fig. 5. Expression of the ECS elements, and origin of AEA/2-AG tone in NP cells. (A) Dynamic regulation of the expression levels of ECS elements along neuronal differentiation during cortical development. (B) Metabolic pathways involved in the synthesis of the eCBs AEA and 2-AG in NP cells. See text for details.

that drive NP proliferation [86,96,97]. In addition, depletion of the AEA tone in the developing cortex interfes with radial migration of NPs [82], a process that is concomitant to (and required for) appropriate neuronal differentiation and acquisition of laminar and projection identity.

Further research is required to elucidate the role of synaptic neuronal activity (in adult neurogenic zones) or spontaneous activity (during brain development) in the regulation of eCB production and NP cell fate. Ionotropic and metabotropic glutamate receptors (mGluR) can engage 2-AG generation via G_q-PLC activation and/or increased Ca^{2+} levels [47], and this may occur in neurotransmitter-mediated, but also in spontaneous neuronal activity during cortical development [98]. mGluR5 activation is a predominant source of 2-AG generation as retrograde neuromodulatory messenger of active synapses, but it is also relevant in somatosensory cortical development and adult hippocampal NP proliferation [99]. Likewise, the other major neurotransmitter GABA regulates adult NP proliferation [100] and during brain development glutamate decarboxylase (GAD) activity, that is responsible for GABA synthesis, is involved in interneuron development and synapse maturation. Noteworthy the ECS, and CB_1 receptors in particular, are associated to regulation of somatosensory cortical development [101], and to interneuron differentiation [69]. As yet, the role of neuronal activity in eCB generation and regulation of NP cells remains to be clarified.

3.2. Cannabinoid receptors in neural progenitor cell proliferation and differentiation

First evidence for an active role of CB receptors in NP cells came from studies on the regulation of adult neurogenesis by cannabinoid administration or genetic ablation of the CB_1 receptor [86,102–104]. These studies evidenced that loss of CB_1 signaling inhibits NP cell proliferation *in vitro* and *in vivo*, as well as reduces the self-renewal ability of NPs. Loss of CB_1 receptor signaling reduced hippocampal and SVZ-NP proliferation in the adult brain [86,103], an effect reminiscent of its positive role in NP proliferation during cortical development [82]. CB_1 signaling also affects postmitotic neuronal differentiation independently of its regulatory role in undifferentiated progenitor cells, as shown by the use of postmitotic glutamatergic neuron-conditional CB_1-deficient mice [70]. On the one hand, CB_1 signaling in NPs engages proliferative and/or prosurvival signaling cascades (Section 3.3) that contribute to the regulation of cell cycle maintenance and to the switch between cell proliferation and differentiation/migration. In addition, CB_1 signaling influences the identity and cell features by regulating neuronal [82,105] and glial differentiation [96,106] (Section 3.4). Findings in the studies reviewed herein have demonstrated the notion that eCB ligands constitute novel signaling cues involved in the regulation of NP cell fate decisions and neurogenesis by regulating cannabinoid receptor activity and distinct eCB-metabolizing enzymes (summarized in Fig. 6).

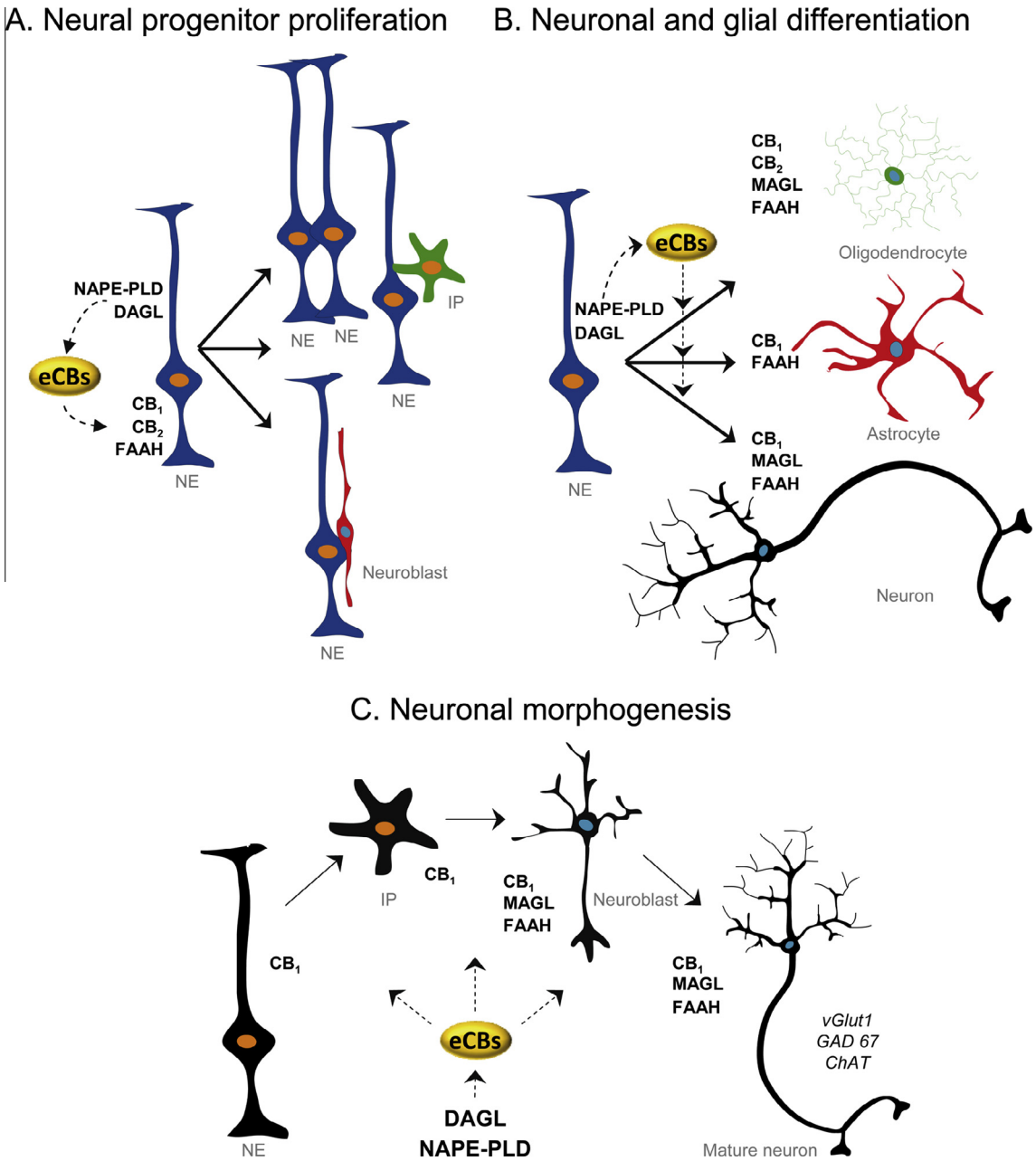

Fig. 6. Summary of cell fate decisions of neural progenitors and differentiating cells regulated by the ECS. The eCB-binding receptors and eCB-metabolic enzymes shown to be involved in the regulation of (A) NP proliferation, (B) neuronal and glial differentiation and (C) neuronal morphogenesis are indicated. Neurochemical markers associated to CB_1 activity in developing neurons are shown in (C). See text for details and abbreviations.

3.3. Cannabinoid receptor signaling in neural progenitor cells

Cannabinoid receptor signaling participates in the cell autonomous control of proliferation and self-renewal of NPs, and several pathways have been implicated in these events. CB_1 and CB_2 are coupled to the activation of the ERK and the PI3K/Akt pathways [14], both of which are classical routes that promote cell survival and proliferation in most biological systems (Fig. 7). CB_1 coupling to heterotrimeric $G_{i/o}$ proteins and the resultant reduction in cyclic AMP levels mediate, at least in part, the activation of the ERK pathway [107–109] by preventing the inhibitory effect of PKA over Raf-1 [110]. Additional mechanisms may take part along the duration/kinetics of CB_1-induced ERK activation including $G_{\beta\gamma}$-dependent $PI3K_{1B}$ activation, as well as the regulation of cytosolic tyrosine kinase Src-family members and membrane receptors of growth factors, neurotrophins and glutamate [109,111]. During interneuron differentiation, CB_1 receptor activity controls growth cone collapse via the monomeric G protein RhoA [81], and migration and neurite morphogenesis via the cytosolic Src-mediated transactivation of TrkB receptors [112].

The proliferative role of the PI3K/Akt cascade in CB_1/CB_2 signaling in NPs has been investigated in detail. In cerebellar progenitor cells CB_1-induced cell proliferation has been shown to be mediated by the PI3K/Akt/GSK3β (glycogen synthase kinase 3β) signaling. As a consequence of GSK3β inhibition, the increased β-catenin nuclear translocation drives cyclin D1 expression [113]. In addition, CB_2-mediated proliferation of NPs involves the PI3K/Akt/mTORC1 (mammalian target of rapamycin complex 1) pathway which, by

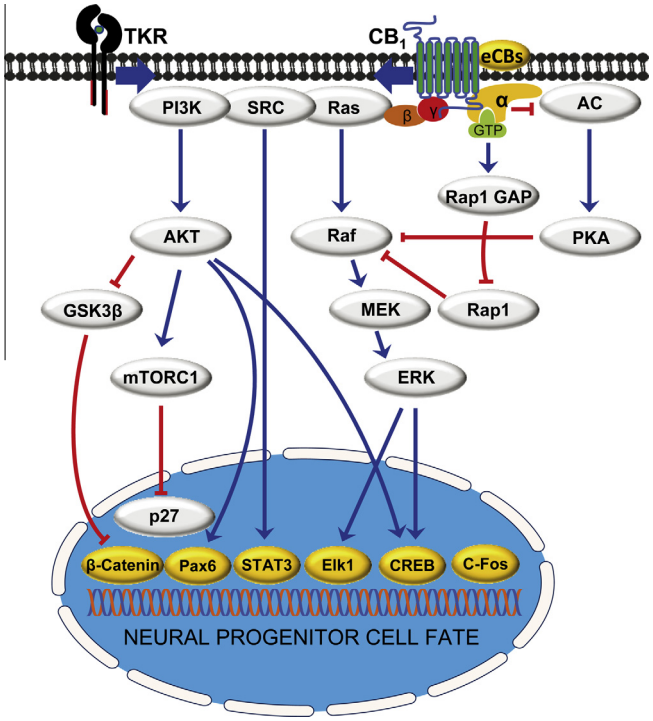

Fig. 7. CB_1 receptor signaling mechanisms in neural cells. See text for details and abbreviations.

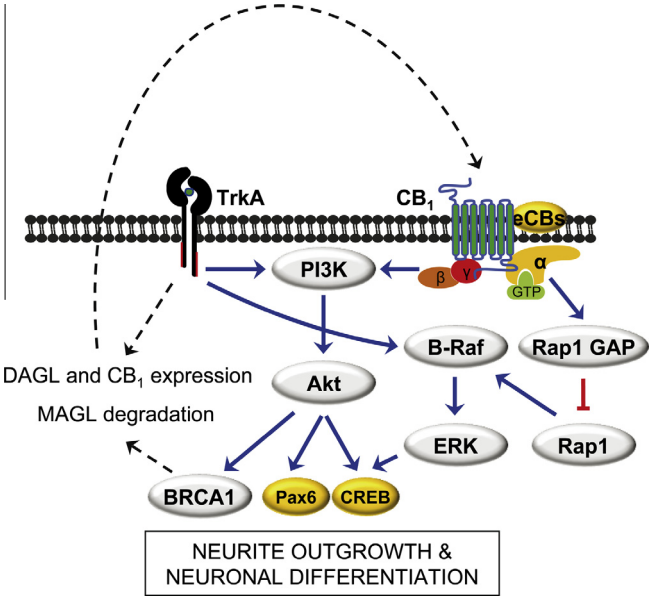

Fig. 8. CB_1 receptor signaling crosstalk with NGF in neuronal differentiation. See text for details and abbreviations.

inhibiting its downstream target $p27^{Kip1}$, induces NP proliferation [75]. Likewise, in cortical progenitors CB_1 drives mTORC1 signaling and cell proliferation (Díaz-Alonso and Galve-Roperh, unpublished). Considering the similarities of CB_1 and CB_2 downstream signaling mechanisms, an attractive hypothesis that requires further investigation is whether homo- or hetero-dimers of CB_1 and CB_2 can tune eCB signaling in NPs. In line with this, in neuroblastoma cells neurite outgrowth is influenced by receptor dimerization and CB_1 and CB_2 activity mutually influences downstream signaling cascades [114].

The emerging scenario is that the CB_1 receptor tunes the NP signalosome of protein kinase cascades involved in the regulation of cell fate decisions. Proliferation and differentiation are mutually excluding cellular processes, and CB_1 signaling is active in both cycling progenitors and after cell cycle exit and differentiation. These findings suggest that CB_1 may engage distinct downstream signal transduction partners, or it may trigger similar signaling pathways but with different efficacy in proliferating progenitors and postmitotic cells, as documented in excitatory and inhibitory neurons [115].

3.4. Cannabinoid receptor regulation of neural cell fate and identity gene expression

CB_1 regulates the expression of genes that determine neural identity and are responsible for the control of cell fate decisions during nervous system development [70,116], although the short-term signaling events (e.g., phosphorylation by protein kinases) involved in gene expression regulation remain largely unexplored.

The homeodomain containing transcription factor Pax6 is involved in NP proliferation and acquisition of VZ apical progenitor identity, and it also influences neuronal differentiation and cortical projection identity. CB_1 activation regulates Pax6 postranslationally by phosphorylation mediated by the PI3K/Akt pathway in differentiating neuroblastoma cells (Fig. 8), and this effect contributes to the kinase signaling and the transcription factor networks involved in neurite outgrowth [116]. In addition, CB_1 increases Pax6 expression in cortical progenitors, driving the expansion towards basal intermediate progenitors by inducing the expression of the transcription factor Tbr2/eomes (Díaz-Alonso and Galve-Roperh, unpublished). Neuronal differentiation occurs in an inside-out patterned process with upper layers generated at later developmental stages than deep layers during corticogenesis [117]. CB_1 regulates the balance of expression of Ctip2 and Satb2, two transcriptional regulators that are involved in the decision switch of deep- *versus* upper-layer cortical neurons. Ctip2 drives deep-layer cortical neuronal identity and corticospinal connectivity, whereas Satb2 is involved in intracortical projection neurons selectively arising from upper cortical layers [117]. Deletion of CB_1 during mouse cortical development lowered Ctip2 expression and generation of deep layer V neurons and these is reflected in the reduced ability for skilled motor activity of CB_1-deficient mice [70].

In postnatal NPs, cannabinoid receptor activity has been shown to regulate astroglial and oligodendroglial differentiation. Thus, CB_1 activity increases astroglial differentiation and GFAP expression in the developing cortex [96,118]. In oligodendrocyte progenitor cells CB_1 and CB_2 activation promotes the expression of Olig-2 in a PI3K/Akt/mTORC1-dependent manner [119].

In addition to the aforementioned cell-autonomous regulation, CB_1 may also regulate neural cell fate decisions through its ability to modulate growth factor, neurotrophin and cytokine expression and, thereby, their selective receptor downstream signaling mechanisms. Transcriptome analysis revealed that whereas CB_1 and CB_2 do not contribute significantly to the global activation of PI3K and ERK signaling pathways in NPs, they regulate a common set of transcripts in cooperation with epidermal growth factor receptors [120]. CB_1 signaling synergizes with other canonical pathways involved in neurochemical specification. For example, CB_1 transactivates BDNF TrkB receptors during interneuron morphogenesis [112]. Additionally, CB_1 regulates BDNF expression [108,121] that, besides exerting a pro-survival action in neuronal cells, can contribute to neuronal projection and interneuron specification [122,123]. Likewise, BDNF expression is involved in excitotoxicity-induced hippocampal NP proliferation mediated by CB_1 [88]. During cholinergic differentiation, NGF upregulates the expression of the ECS

signaling elements CB_1, DAGL and MAGL, and the control of the subcellular localization of MAGL and DAGL via Brca1 E3-ubiquitin ligase activity regulates neurite morphogenesis (Fig. 8) [80]. Increased levels of 2-AG, observed in NGF-induced differentiating cells, can in turn induce a feedback regulatory mechanism by activating CB_1 that controls the activation of the Rap1/B-Raf upstream branch of ERK activation induced by NGF/TrkA responsible for neurite outgrowth [102]. In addition, CB_1 can attenuate growth factor/neurotrophin receptor signaling by releasing Sprouty, an inhibitor of the MAPK pathway, from "G protein-regulated inducer of neurite outgrowth" GRIN [124]. These and most likely other yet unknown crosstalk mechanisms add to the direct role of CB_1 signaling in neurite outgrowth via PI3K/Akt-mediated control of Pax6 in NPs (Figs. 7 and 8).

3.5. Type-1 cannabinoid receptor regulates neuronal morphogenesis and differentiation

CB_1 receptor located in axon growth cones of differentiating neurons induces a collapse response by sensing DAGL-derived 2-AG, which requires a tight spatio-temporal tuning of 2-AG availability [81,82,125,126]. The balance between DAGL and MAGL activities regulates subcellular levels of 2-AG in motile neurites of cortical projection, retinal, GABAergic, and cholinergic neurons. Indeed, MAGL is enriched in tubulin-consolidating axon shafts while DAGL accumulates in actin-rich motile axon tips, thus generating a 2-AG gradient that triggers axonal growth cone collapse. In cortical and retinal neurons CB_1 regulates axonal growth cone by controlling the plasma membrane localization of the Dcc (deleted in colorectal cancer) receptor [126], whereas in GABAergic interneurons the monomeric G protein RhoA is involved [81].

Likewise, the establishment of long-range subcortical projections is regulated by CB_1 signaling during development and, thus, ablation or pharmacological blockade of CB_1 receptors *in utero* alters corticothalamic projections and induces axon fasciculation deficits [78,82]. The complementary expression pattern of DAGL in thalamocortical axons and of MAGL in corticothalamic and thalamocortical developing axons may contribute to the generation of spatially-restricted 2-AG pools. It has therefore been suggested a potential role for 2-AG as one of the molecules responsible for the timely developmental coordination between corticothalamic and thalamocortical projection "hand-shaking" [125,127]. CB_1 thus exerts an acute/short-term regulation of growth cone signaling in neurite tips, as well as long lasting changes in neurogenic gene expression that affect neuronal wiring and connectivity overall. In agreement with these observations, pharmacological blockade of CB_1 interferes with spinal cord axon growth and fasciculation [78]. The axonal pool of CB_1 receptors during embryonic development overlaps with L1-CAM (L1-cell adhesion molecule), and CB_1 may alter the expression of this protein in differentiating neurons [128]. The Pax6 transcription factor is a plausible candidate to mediate these actions, as the L1-CAM gene promoter is regulated by Pax6 in an mTORC1-dependent manner [129]. Reciprocally, 2-AG generation and CB_1 signaling are required for the axonal growth response to NCAM adhesion molecules and FGF receptor activation [93]. In early embryonic chick development, administration of a THC analog disrupts neurogenesis and affects brain, somite and spinal cord primordium development, suggesting that the ECS may be active in early cell fate decisions of the progenitor cells involved in the formation of the neural tube [130].

3.6. Developmental implications of cannabinoid receptor signaling in neural progenitor cells

The neurodevelopmental role of the ECS and its ability to regulate NP cell fate can induce altered function of the adult nervous system, with important implications in distinct pathophysiological circumstances. The appropriate balance of excitatory and inhibitory cell populations generated during brain development is essential for coordinated neuronal activity, and unbalanced neurogenesis of these neuronal lineages can originate excessive neuronal activity. Thus, regulation of excitatory projection specification and long range connectivity, and of GABAergic interneuron morphogenesis and migration by CB_1 [69,105], can underlie the higher susceptibility to seizure and severity in CB_1-deficient mice [131]. In addition, embryonic loss of CB_1 signaling has been shown to exert long-lasting alterations in neuronal differentiation that influence motor activity in the adult brain [70]. Subtle alterations in neuronal number by defective CB_1 activity or developmental exposure to phytocannabinoids may also be induced by alterations of NP cell proliferation and cell cycle control [82,86]. However, the neurobiological substrate responsible for the alteration of the control of emotions, social interaction and cognitive aspects induced by phytocannabinoid consumption [132], or by an unbalanced eCB signaling [133,134] during brain development, remains to be investigated. Furthermore, the role of CB_1 in alterations of interneuron development underlying the pathogenesis of schizophrenia constitutes an expanding field of research [84]. The reviewed studies of cannabinoid receptor regulation of NP cell proliferation and differentiation underscore the deleterious consequences of phytocannabinoid exposure during development [135], but they also raise hope for new therapeutic avenues in psychiatric and neurodegenerative disorders of developmental origin. Finally, as discussed in the "Conclusions and future directions" section, regulation of progenitor/stem cells and adult neurogenesis by eCB signaling also opens new vistas of potential regulation of NP cells under pathophysiological circumstances.

4. Cannabinoid receptor regulation of peripheral cell differentiation

4.1. Regulation of hematopoietic cell differentiation

Hematopoiesis is a tightly regulated process that allows to both maintain physiological levels of cells and respond to pathological conditions. Stem cell migration is a common feature of hematopoiesis, and it occurs during development and throughout life. Indeed, hematopoietic progenitor/stem cells (HSC) continuously traffic from (mobilization) and to (homing) their bone marrow niche [136]. These trafficking processes, especially the egress of HSC from bone marrow, are regulated by various agents, including cytokines and chemotherapeutics [137,138], and more recently also eCBs (Fig. 9). In this context, AEA and 2-AG stimulate hamatopoiesis and HSC migration, respectively, by synergizing with colony-stimulating factor (CSF), interleukin-3 (IL-3) and erythropoietin through CB_2 [139,140]. 2-AG has also been shown to increase CFU-GEMM (colony-forming unit: granulocyte, erythrocyte, macrophage, megakaryocyte)-induced colony formation and cell migration in a CB_1- and CB_2-dependent manner, whereas AEA increases colony yield but inhibited cell migration via CB_1 and CB_2 [141]. Furthermore, activation of both CB receptors in murine ES cells has been shown to promote hematopoietic differentiation [56]. CB_2 regulates bone marrow myeloid progenitor trafficking by altering the expression of chemokines and of their receptors [142]. In line with this, early hematopoietic stem cells treated with the CB_1 and CB_2 agonist CP55940 show significant reduction in both CXCR4 and VLA-4, suggesting that such receptors might be involved in the release of HSCs from bone marrow niches [143]. Additional data suggest that particularly CB_2 activation rapidly mobilizes CFU-GM and enhances mobilization by G-CSF, likely through inhibition of CXCR4 signaling or of integrin adherence. Furthermore, CB_2 agonism promotes bone

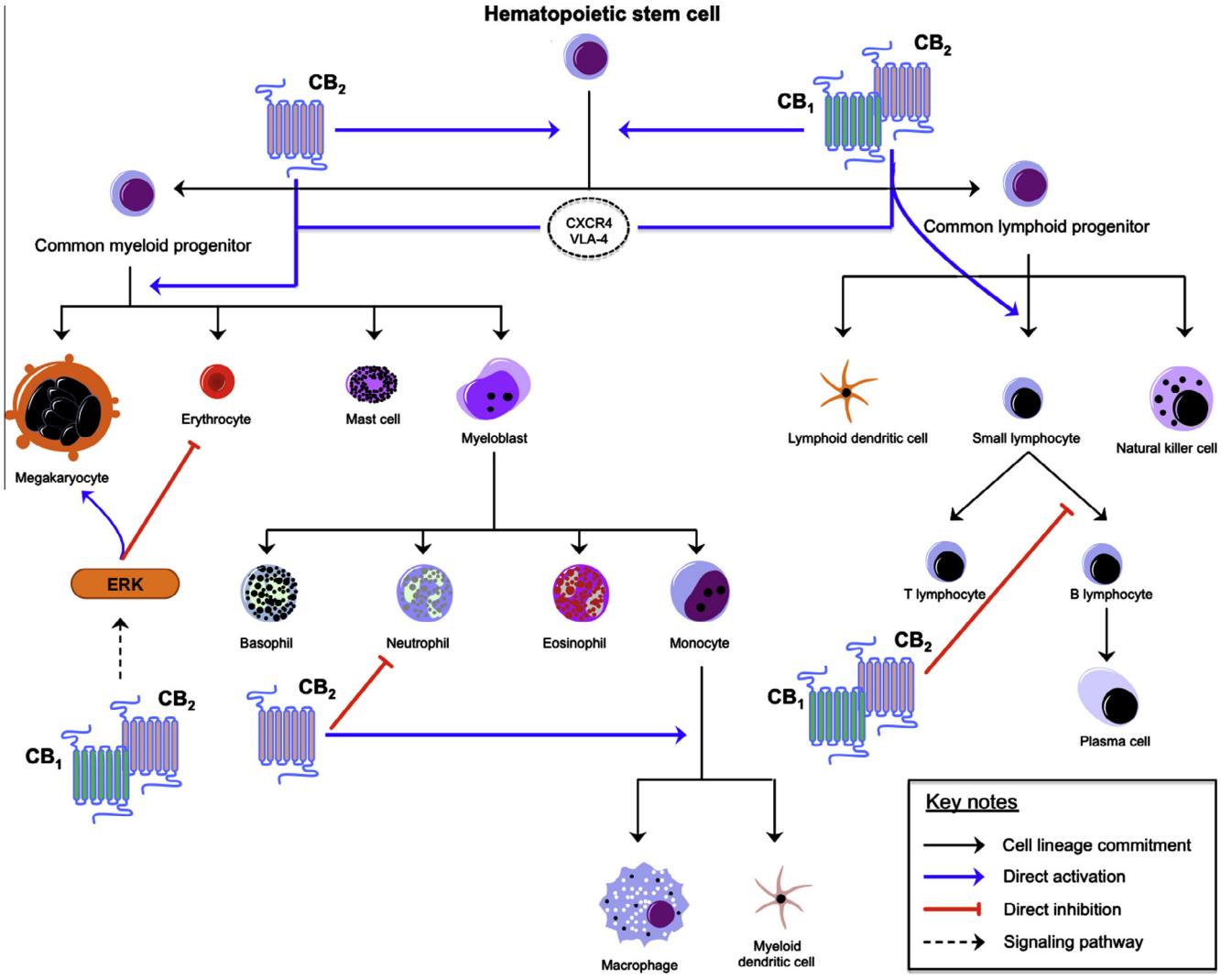

Fig. 9. Cannabinoid signaling in the control of cell lineage commitment during hematopoiesis and differentiation of distinct immune cell populations. See text for details and abbreviations.

marrow recovery and increases total marrow cells following sublethal irradiation, by inhibiting apoptosis and hence promoting survival of HSCs, as well as by increasing the number of HSCs that enter cell cycle [144]. It should be noted that these effects of eCBs on CFU-GM mobilization are in contrast with a study showing that CB_2 mediates retention of immature B cells in bone marrow sinusoids [145], suggesting a possible cell-type specificity of mobilization responses. While these studies clearly indicate mobilization of HSCs, further analysis of the full repertoire of these cells mobilized by eCBs might support the development of novel cannabinoid receptor-based mobilization strategies. In addition, 2-AG and CB_1/CB_2 stimulation increases fibroblastic colony formation and colony size, and it recruits mesenchymal stem cells from the bone marrow, most probably via an indirect activation of CB_2 receptors [146]. Moreover, 2-AG drives human erythroleukemia (HEL) cells towards megakaryocytic differentiation by enhancing the expression of β3 integrin subunit (a megakaryocyte/platelet surface antigen), and of glycoprotein VI (a late marker of megakaryocytes). In parallel, 2-AG reduces the transcription of glycophorin A, a marker of erythroid phenotype [147]. All these effects are mediated by activation of CB_2, which triggers an ERK-dependent signaling cascade. Remarkably, classical inducers of megakaryocyte differentiation reduced 2-AG synthesis, suggesting that levels of this eCB may be critical for committing HEL cells towards the megakaryocytic lineage [147].

4.2. Regulation of immune cell differentiation

Immune cells mainly express CB_2, with NK cells, monocytes/macrophages and B-lymphocytes bearing the highest levels compared to neutrophils and resting CD4 or CD8 T-lymphocytes [148]. The first evidence of a possible regulation of immune cell differentiation by the ECS came for the study by Murison and colleagues, who showed that several phytocannabinoids induced the expression of monocyte maturation markers in human ML-2 leukemia cells, and a modulation of CB_2 during the differentiation stages of B-lymphocytes. Yet, these compounds failed to sustain terminal cell differentiation [149]. Indeed, a clear downregulation of CB_2 expression during B-cell differentiation, as well as a CB_2-mediated increase in cell proliferation of both CD40-activated B cell subsets, were documented, strongly supporting an engagement of CB_2 during B-cell differentiation [150]. Furthermore, T cell differentiation in the neonatal and postnatal stages of life has been shown to be profoundly altered by CB_1 and CB_2 signaling, with a major impact on susceptibility to infections and other immune disorders [151].

As far as innate immune cells are concerned, most of the available data are focused on the monocyte-to-macrophage/neutrophil differentiation model, in particular using two human monocytic cell lines, HL-60 and U937. In this context, 2-AG was found to induce rapid actin polymerization and pseudopod extension in

differentiated HL-60 cells in a CB_2-dependent manner that involves the PI3K pathway [152,153]. However, this result is at variance with the more recent evidence that WIN55212-2, a CB_1 and CB_2 mixed agonist, inhibited the CB_1/CB_2-dependent differentiation of human myelocytic U937 cells into a macrophageal phenotype; such a differentiation was associated with impaired vimentin, intercellular adhesion molecule 1 (ICAM-1) and CD11b expression, as well as with altered cell cycle control proteins cdc2 and p21 [154]. Interestingly, Affymetrix analysis of gene expression profile of HL-60 cells revealed that CB_2 induced the activation of several genes involved in cell differentiation, suggesting that the latter receptors could play a role in the initialization of cell maturation. Moreover, CB_2-activated wild-type HL-60 cells acquired features that are usually found in host defense effector cells, such as enhanced release of chemotactic cytokines and increased motility, and that are typical of more mature cells of the granulocytic-monocytic lineage [155]. Consistently, CB_2 has been shown to regulate excessive inflammatory response *in vivo* by controlling RhoA activation, thereby suppressing neutrophil migration [156].

A very recent and interesting investigation has documented an unprecedented role of eCB signaling in the maturation of mast cells [157]. The latter are resident in many tissues derived from the bone marrow, and play a major protective role in wound healing and defense against pathogens, especially within connective and mucosal tissues [158]. It was demonstrated that mast cells in normal human skin utilize CB_1-mediated signaling to limit not only their own activation/degranulation, but also their maturation from resident progenitor cells *in situ* [157]. Additionally, CB_1 and CB_2 have also a role in the regulation of the newly characterized myeloid-derived suppressor cells, which are a heterogeneous population of immature hematopoietic precursors, known to suppress immune responses during infection, chronic inflammation, cancer, and autoimmunity [159]. Activation of both CB_1 and CB_2 receptors elicited a massive mobilization of these cells in mice, where they exhibited potent immunosuppressive properties, both *in vitro* and *in vivo*, according to a G-CSF-dependent mechanism [160]. The main effects of the ECS on the different populations of immune cells are summarized in Fig. 9.

4.3. Regulation of adipocyte differentiation

The growth of adipose tissue involves the increase in adipocyte size and the formation of new adipocytes from precursor cells. Committed pre-adipocytes undergo growth arrest and subsequent terminal differentiation into adipocytes, and these steps are followed by a dramatic upregulation of adipocyte specific gene expression [161]. Growth and differentiation of pre-adipocytes are controlled by communication between individual cells and/or between cells and the surrounding extracellular environment [161]. Human adipose tissue possesses CB receptors and a functional ECS [162,163]. Noteworthy, CB_1 receptor expression has been found to change along adipocyte differentiation, being low at the pre-adipocyte stage and high when the adipocytes become mature [164–167]. CB_1 stimulates adipocyte proliferation and its antagonism with SR141716 (rimonabant) inhibits adipocyte cell growth and stimulates mRNA and protein expression of two late markers of adipocyte maturation (adiponectin and glyceraldehyde-3-phosphate dehydrogenase) in cultured preadipocytes, giving a molecular hint to the rimonabant-induced anti-obesity effects like the reduction of body fat mass [168,169]. Also AEA has been shown to promote adipocyte differentiation through either CB_1 signaling or transcriptional activation of PPARγ, a well-recognized marker of adipogenesis [163,170,171]. In addition, a key process in maintenance of energy balance and body weight is glucose transport [161]. Interestingly, AEA has been shown to increase insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes by a CB_1- and nitric oxide synthase-dependent mechanism [163]. In line with these observations, eCBs were also found to possess an insulin-mimetic action on glucose uptake, which was mediated by activation of CB_1 in human adipose tissue [167].

It is well-known that CB_1 modulation in adipose tissue, both *in vitro* and *in vivo*, is able to modify adipokine synthesis and production. Indeed, CB_1 blockade increases adiponectin in white adipose tissue and 3T3-F44A adipocytes [162,164,172], whereas its activation reduces mRNA levels of adiponectin [162,173] and increases those of visfatin [173]. Furthermore, Bellocchio and colleagues were the first to describe a CB_1- and CB_2-dependent upregulation of the novel adipokine apeline in mature adipocytes [169], although only CB_1 activation potently stimulated lipogenesis in adipocytes, by increasing lipid metabolism and promoting intracellular fat content and changes in lipid composition [169,174]. Recently, an interesting study documented a crosstalk between gut microbiota and the regulation of adipogenesis by the ECS, where the former modulated CB_1 both in the intestine (where it led to increased gut permeability), and in the adipose tissue (where it controlled adipogenesis and fat mass accumulation). In turn, peripheral CB_1 controlled gut barrier function and adipogenesis [175]. A schematic representation of the most important functions of eCB signaling in adipocyte differentiation is shown in Fig. 10.

4.4. Regulation of bone turnover

Bone remodeling is a delicate balance between removal and replacement of bone mass by dedicated cell types, the osteoclasts and osteoblasts respectively [176,177]. Recent studies have shown that both CB_1 and CB_2 receptors play distinct roles in regulating bone mass and bone turnover (summarized in Fig. 10). Genetic inactivation or pharmacological inhibition of CB_1 determines a defect in osteoclast differentiation and resistance to ovariectomy-induced bone loss [178], and these effects were related to a reduction in the sensitivity of osteoclast precursors to RANKL (receptor activator of nuclear factor kappa-B ligand), or to a reduction in RANKL expression in osteoblasts [179].

The equilibrium between bone resorption and bone formation is also the hallmark of osteoporosis, where bone mesenchymal stem cells from elderly subjects have a reduced capacity to differentiate into osteoblasts and an increased capacity to differentiate into adipocytes, *via* a CREB (cAMP response element-binding protein)- and PPAR-mediated mechanism, ultimately leading to progressive accumulation of fat in the bone marrow compartment [180]. This process seems to be regulated by CB_1 signaling. Indeed, deficiency of CB_1 or its pharmacological blockade enhanced the capacity of adipocytes to differentiate while reducing that of osteoblasts, and increased phosphorylated CREB and PPARγ expression in osteoblast and adipocyte precursors. CB_1 is therefore unique in that it regulates peak bone mass through an effect on osteoclast activity, but it protects against age-related bone loss by regulating adipocyte and osteoblast differentiation of bone marrow stromal cells [179]. Furthermore, it has been shown that CB_1 plays a key role in regulating the increased bone formation following traumatic brain injury, since such an increase was present only in wild-type and $CB_2^{-/-}$ mice. Instead, $CB_1^{-/-}$ mice showed a low bone mass associated with a decreased bone formation rate and mineral apposition, as well as an increased number of osteoclasts [181]. It was also reported that traumatic brain injury-induced bone formation in wild-type mice was abolished by the β-adrenergic agonist isoproterenol, suggesting that CB_1 at presynaptic nerve endings might enhance bone formation by suppressing catecholamine release [181].

The role of CB_2 in the regulation of bone metabolism is still a matter of debate. The first evidence documented a low bone mass phenotype in mice deficient for CB_2, along with an enhanced

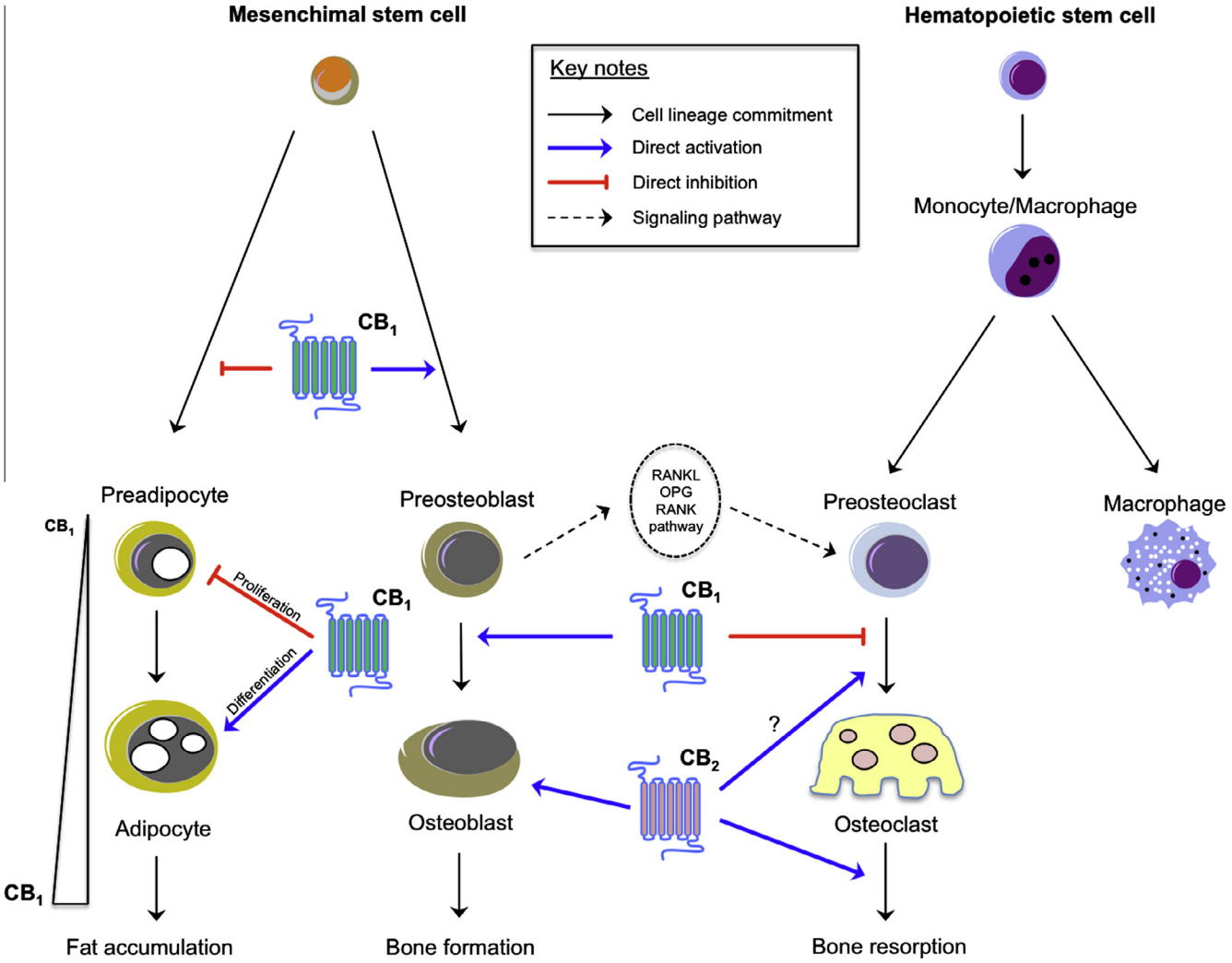

Fig. 10. Cannabinoid signaling in the control of cell proliferation and differentiation of adipocytes and bone tissue, and their cross-talk. See text for details and abbreviations.

endocortical osteoblast number and activity and restrained trabecular osteoclastogenesis, both mediated by the latter receptor [182]. Yet, Idris and co-workers reported that CB_2 activation increased M-CSF- and RANKL-induced osteoclast formation and activity *in vitro*, and that CB_2 selective antagonism inhibited osteoclast formation. Overall, it seems that CB_2 may regulate osteoclast formation and bone resorption *in vitro*, and that under conditions of increased bone turnover, such as after ovariectomy, it may regulate bone loss [178,183]. This view is further supported by the evidence that pharmacological blockage of CB_2 negatively regulates RANKL-mediated osteoclastogenesis, in terms of reduction of osteoclast formation and expression of osteoclast differentiation/activation markers [184].

CB_2 also plays a key role in regulating bone formation. Indeed, several CB_2 selective agonists have been shown to stimulate bone nodule formation in bone marrow stromal cell cultures *in vitro*, although similar effects have been observed also with non-selective agonists, including eCBs and synthetic cannabinoids

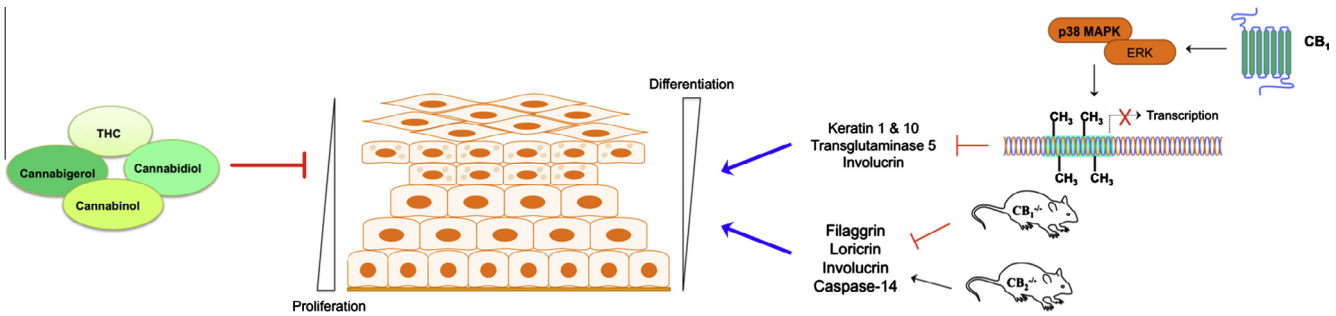

Fig. 11. Cannabinoid signaling in the control of epidermal differentiation. See text for details and abbreviations.

[146,179,182]. A specific role for CB_2 is supported by the observation that bone marrow stromal cells from $CB_2^{-/-}$ mice have a reduced capacity to differentiate into bone nodules. Furthermore, although $CB_2^{-/-}$ mice have increased bone turnover, there is a relative defect in bone formation, as shown by the fact that $CB_2^{-/-}$ mice develop age-related osteoporosis [182].

4.5. Control of epidermal differentiation

The epidermis, which forms the uppermost compartment of the skin, represents a barrier against the environment, provided by terminally differentiating keratinocytes [185,186]. Epidermal differentiation begins with keratinocyte migration from the basal layer composed of proliferating cells, and it ends with the formation of the cornified cell envelope, an insoluble protein structure found in differentiated keratinocytes [187]. Cell proliferation and differentiation occur sequentially and are characterized by the expression of specific proteins, such as keratins and transglutaminases [188,189]. The role of the ECS in the control of epidermal differentiation was mainly investigated by some of us, and the first evidence was that AEA inhibited the differentiation of human keratinocytes via CB_1 activation [190]. Furthermore, we disclosed some molecular details responsible for this effect, demonstrating that keratin 1 and 10, transglutaminase 5 and involucrin were all transcriptionally downregulated by AEA [191]. This effect of AEA was due to increased DNA methylation through a p38 mitogen-activated protein kinase- and to a lesser extent ERK-dependent pathway triggered by CB_1. However, several phytocannabinoids were found to inhibit keratinocyte proliferation in a non-CB_1/CB_2-dependent manner [192] (Fig. 11). The specific role of CB_1 and CB_2 receptors in keratinocyte proliferation/differentiation was recently addressed by Roelandt and colleagues, who demonstrated that CB_1 and CB_2 have opposite effects in controlling the epidermal permeability barrier and keratinocyte differentiation, with CB_1 promoting epidermal and terminal differentiation and CB_2 being ineffective [193]. These discrepancies in the literature are schematically illustrated in Fig. 11, and highlight the importance and complexity of eCB signaling in the control of epidermal differentiation. They also call for further investigation aimed at better understanding how cannabinoid receptors might be targeted in those pathologies linked with abnormal epidermis differentiation or altered epidermal permeability barrier function.

5. Conclusions and future directions

The last decade has provided unexpected findings that document the role of cannabinoid receptors in regulating ectoderm-derived neural progenitor/stem cell and mesoderm-derived hematopoietic progenitor/stem cell fate decisions. eCB signaling in progenitor/stem cell niches regulates cell proliferation and tunes the differentiation profile of progenitor-derived daughter cells. CB_1 and CB_2 activation both increases cell proliferation and expands the available NP cell population. Neuronal and glial specification is differentially regulated by CB_1, which, according to the cellular context, may drive neuronal or glial differentiation. Instead, CB_2 is downregulated along neuronal differentiation, and its potential regulatory role in neural differentiation is probably associated with injury responses. A prominent role for CB_2 in regulating non-neural progenitor cells, such as immune cell differentiation, haematopoiesis and bone remodeling, has been documented. In humans, its relevance may be exemplified in abnormal blood cell development. In particular, CB_2 is expressed in acute myeloid leukemia blast cells from patients, but not in myeloid cells from healthy subjects, and its overexpression in myeloid precursor cells leads to ERK- and PI3K-dependent abnormal migration or blockade of neutrophil differentiation [194,195].

NPs residing in restricted neurogenic areas of the mammalian adult brain respond to neuronal insults, induced by either brain injury or neurodegenerative disorders, with an increased mobilization that leads to the generation of newly born neurons; the latter can then integrate in pre-existing neuronal circuits. Thus, injury-induced neurogenesis (i.e., after seizure or stroke) may provide some benefit and attenuate the consequences of neuronal loss. Alternatively, neurogenesis may also contribute to neuronal plasticity mal-adaptations after injury (mossy fiber sprouting, ectopic migration and altered excitability) that contribute to the development of epileptogenesis [196]. Future research aimed at deciphering the characteristics and functionality of CB_1 and CB_2 receptor-induced hippocampal NP proliferation [75,88] is required to determine whether CB_1 and CB_2 activation or blockade may be of therapeutic value to modulate hippocampal neurogenesis after injury. Likewise, 2-AG, via CB_1 and CB_2, regulates SVZ-derived NP proliferation [74,103] and may act as a migration regulatory cue for neuroblasts, thus influencing the ability of newly born cells to reach the olfactory bulb along the rostral migratory stream [87]. It remains, however, to be clarified whether eCB signaling contributes to the directionality of SVZ-derived neuroblasts at the sites of injury. Importantly, eCB synthesis by NPs can contribute to the neuroprotective role of the ECS, and, in a model of stroke and seizure-induced NP mobilization and AEA generation, spontaneous excitatory postsynaptic currents, striatal neurodegeneration, and mice mortality was shown to be increased by NP ablation [197]. Thus, eCB production after brain injury and in neurodegenerative disorders exerts a neuroprotective action that includes: (i) cell autonomous neuroprotection via CB_1 [198]; (ii) regulation of NP proliferation and neuronal differentiation via CB_1 and/or CB_2 in either neuronal or glial cell lineages; and iii) regulation of the neuroinflammatory environment by modulating the activation and migration of peripheral immune cells [20], resident microglia/macrophages [199], and their progenitors [142].

Hippocampal neurogenesis contributes to different aspects of cognition and emotional states, and the potential therapeutic use of neurogenesis-boosting strategies in mood disorders, such as depression, is a challenging opportunity [200]. Whereas the negative consequences of plant-derived, synthetic or endogenous cannabinoids in the regulation of emotional states and cognition are well-known [72], under some circumstances cannabinoid compounds can exert cognitive and anxiolytic actions that may be beneficial. Chronic injection of a CB_1 agonist to mice exerted an anxiolytic action by increasing hippocampal neurogenesis, and these findings were replicated by a non-psychomimetic-cannabinoid administration paradigm [97,104]. Likewise, a CB_1 and CB_2 mixed agonist palliated ageing-associated decline of mouse neurogenesis [74], although its impact on cognitive functions remains unknown. Overall, further research on the precise roles of eCB signaling in progenitor/stem cell biology may provide in the next future new grounds for understanding the therapeutic potential of cannabinoid-based treatments.

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